Eesti Maaülikool
Estonian University of Life Sciences

GENETIC DIVERSITY, GENOTYPIC STRUCTURE AND VULNERABILITY OF NATIVE POPULATIONS OF SICKLE MEDIC (*MEDICAGO SATIVA* SSP. *FALCATA*) IN ESTONIA

SIRPLUTSERNI (*MEDICAGO SATIVA* SSP. *FALCATA*) LOODUSLIKE POPULATSIOONIDE GENEETILINE MITMEKESISUS, GENOTÜÜPNE STRUKTUUR JA OHUSTATUS EESTIS

KARIN KALJUND

A Thesis for applying for the degree of Doctor of Philosophy in Botany

Väitekiri filosoofiadoktori kraadi taotlemiseks botaanika erialal

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Opponent: Dr. Jan Kirschner
Institute of Botany
Academy of Sciences of the Czech Republic

Supervisors: Dr. Malle Leht
Institute of Agricultural and Environmental Sciences
Estonian University of Life Sciences

Dr. Vello Jaaska
Institute of Agricultural and Environmental Sciences
Estonian University of Life Sciences

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals in the text. The papers are reproduced by kind permission of the publishers.

I **Kaljund, K.**, Leht, M. 2013. Extensive introgressive hybridization from cultivated lucerne to populations of native sickle medic (*Medicago sativa* ssp. *falcata*) in Estonia. Annales Botanici Fennici 50: 23-31.

II **Kaljund K.**, Jaaska, V. 2010. No loss of genetic diversity in small and isolated populations of *Medicago sativa* subsp. *falcata.* Biochemical Systematics and Ecology 38: 510-520.

III **Kaljund, K.**, Leht, M., Jaaska, V. 2013. Highly variable clonal diversity and spatial structure in populations of sickle medic. Biochemical Systematics and Ecology 47: 93-100.

The contributions of the authors to the papers:

Paper	Original idea and study design	Data collection	Data analyses	Manuscript preparation
I	ML, KK	KK, ML	KK	All
II	VJ, KK	KK, ML	KK, VJ	All
III	VJ, KK	KK, ML, VJ	KK, VJ	All

KK – Karin Kaljund, ML – Malle Leht, VJ – Vello Jaaska

1. INTRODUCTION

The present thesis addresses three important problems of plant population biology and ecology by studying Estonian populations of sickle medic (*Medicago sativa* ssp. *falcata*) (hereafter *falcata*), as a model plant occurring here at the northern periphery of its distribution range. The first problem is related to the contamination and subsequent introgressive hybridization of native Estonian populations of *falcata* by the two introduced cultivated subspecies, ssp. *sativa* and ssp. *varia*. This is a potential threat to the maintenance of native *falcata* populations with their historically adapted gene pools. The gene flow and introgression from domesticated plants into their wild relatives has been extensively studied since the 1970s on the example of numerous crop-wild pairs because of its impact on the fate of wild populations, up to their extinction or the formation of invasive weedy populations (reviewed by Levin *et al*. 1996, Rhymer and Simberloff 1996, Ellstrand *et al*. 1999, Ellstrand and Schierenbeck 2000, Ellstrand 2003, Ellstrand *et al*. 2010). Natural populations of wild relatives of cultivated plants with their unique gene pools, especially those adapted to the adverse conditions of the northern periphery, as are Estonian *falcata* populations, deserve special attention because they may offer valuable genetic material for plant breeding.

The second problem concerned relationships between habitat fragmentation and genetic diversity. Numerous studies have shown that habitat fragmentation presents an increasing threat to the persistence of native populations by lowering their genetic diversity (reviewed by Young *et al*. 1996, Frankham 1996, Montgomery *et al*. 2000, Honnay and Jacquemyn 2007). However, the assumptions about the negative effects of fragmentation seem not always to be valid, and the loss of genetic diversity due to fragmentation appears to vary between different plant species (Leimu *et al*. 2006, Aguilar *et al*. 2008). This indicates a need to study the association between population size and genetic diversity separately for each species by taking into account the species characteristics.

The Estonian *falcata* is known as a clonal plant that combines sexual reproduction via seed progeny with vegetative spread by rhizomes. The third problem addresses relationships between clonal and sexual reproduction. Clonal growth is considered one of the factors improving

the persistence of plant populations, especially in the adverse conditions of northern peripheral areas (reviewed by Klimeš *et al*. 1997, Silvertown 2008). Therefore it is also important to be aware of the genotypic diversity of clonal plant populations, which reflects the overall and spatial distribution of genetic diversity between and within populations (Widén *et al*. 1994, Honnay and Jacquemyn 2008). Genotypic diversity and spatial structure of clonal plant populations varies widely between plant species, depending on the species-specific traits, biological characteristics (e.g., mating system, life history traits, etc.), and habitat conditions (Hamrick *et al*. 1979, Loveless and Hamrick 1984). This indicates a need to study the characteristics of genotypic diversity and spatial structure of populations separately for each clonal species, particularly in relation to the diverse environmental conditions between and within populations.

2. REVIEW OF THE LITERATURE

2.1. The *Medicago sativa-falcata* complex

Hybridization between domesticated taxa and their wild relatives has been observed to be quite common under natural conditions in areas of their sympatric distribution. Today we have evidence that at least 48 cultivated plant species mate with one or more of their wild relatives somewhere in their sympatric distribution area (Ellstrand 2003). In some cases hybridization is a rather rare event without any significant consequences. But among cross-pollinators crop-to-wild hybridization may be quite extensive, for example in lucerne (*Medicago sativa* L), maize (*Zea mays* ssp. *mays*), apple (*Malus domestica* Borkh.), beet (*Beta vulgaris* ssp. *vulgaris* L), cotton (*Gossypium hirsutum* L), radish (*Raphanus sativus* L), carrot (*Daucus carota* L. subsp. *sativus*), rye (*Secale sereale* L), sunflower (*Helianthus annuus* L), oilseed rape (*Brassica napus* L ssp. *napus*), lettuce (*Lactuca sativa* L.) (Ellstrand *et al*. 1999). Likelihood of gene flow for each crop-weed or crop-wild relative combination is different and depends on many factors (Lane 2005). Therefore, for successful hybrid formation some important assumptions must be fulfilled. The crop and its wild relative should grow close to each other in the same region, they should share the same pollinators, their flowering times should be overlapping, and their hybrids have to be viable and fertile (Chapman and Burke 2006).

The *Medicago sativa-falcata* complex is an excellent example for studying hybridization between a crop and its wild form. Taxa belonging to this complex are freely crossing with each other. *Medicago sativa* ssp. *falcata* (L.) Arcangeli (=*M. falcata* L., sickle medic, hereafter *falcata*) is characterised by yellow flowers and sickle-shaped pods, while *M. sativa* ssp. *sativa* L. (hereafter *sativa*) has purple flowers and highly coiled pods (Lesins and Lesins 1979). Their hybrid is called *M. sativa* ssp. x *varia* (Martyn) Arcang. (hereafter *varia*); it also has coiled pods but its flower colour is variegated. The flowers can be mixed purple, yellow, whitish, and greenish, even brown (Small and Brookes 1984). On the same ploidy level the three taxa mate freely producing fertile hybrids and are therefore treated as subspecies of *M. sativa* s.l. (Small and Brookes 1984, Quiros and Bauchan 1988).

Much attention has been paid to hybridization between native and cultivated forms of *sativa* in Spain (Jenczewski *et al*. 1999a, 1999b, Muller *et al*. 2001, Prosperi *et al*. 2006) and in Kazakhstan (Greene *et al*. 2008). The problem of hybridization of native *falcata* with cultivated *sativa* and *varia* has been highlighted in Switzerland (Rufener Al Mazyad and Ammann 1999) and in Germany (Bleeker *et al*. 2007). In Estonia, *falcata* is a native taxon that is growing here on the northern edge of its distribution area. *M*. *sativa* ssp. *sativa* was introduced to Estonia in the 1830ies (Miljan 1932). It has escaped from cultivated fields and has now become partly naturalized, growing scattered all over Estonia, but more often in Western Estonia (Kukk and Kull 2005). The introduced *varia* is more successfully adapted to local natural conditions and is distributed in all parts of Estonia (Kukk 1999, Kukk and Kull 2005). During the 150-year cultivation period, ample diverse cultivars of *sativa* and *varia* have been introduced to Estonia, and have been able to cross with native *falcata*. The first notes about hybrid plants in natural populations of *falcata* were published in the 1970s and 1990s (Bender and Tamm 1998).

2.2. Genetic variation

As natural populations of wild relatives of crop plants offer valuable genetic material for plant breeding, the need for preserving genetically unique natural populations is increasing, especially when preserved genetically pure populations are under pressure from habitat fragmentation and destruction, which are considered to be the main factors reducing biological diversity. Due to decreasing habitat size and increasing isolation distance, small and isolated populations face negative effects such as genetic drift, accompanied by a decrease of genetic diversity and increased influences of inbreeding depression. Therefore several studies have paid a lot of attention to relationships between population size, degree of isolation and genetic diversity, and the results are discussed in several review papers (Young *et al*. 1996, Frankham 1996, Aguilar *et al*. 2008, Honnay and Jacquemyn 2007, Leimu *et al*. 2006, Montgomery *et al*. 2000, and references therein). However, the assumptions about the negative effects of fragmentation on smaller and isolated populations seem not always to be valid, and the loss of genetic diversity due to fragmentation appears to vary among plant species. A number of studies have discovered a positive correlation between fragmentation and reduced genetic diversity, as predicted by the

population genetic theory, especially for short-living annuals (Honnay and Jacquemyn 2007, Leimu *et al*. 2006). However, several works, on the contrary, have not observed any loss of genetic diversity in small and isolated populations of herbaceous perennials (Young *et al*. 1993, 1999, Pluess and Stöcklin 2004, Kuss *et al*. 2008), as well as in shrubs and trees (Prober and Brown 1994). These works indicate that genetic diversity in fragmented populations may be influenced by certain other factors besides the population size and isolation, like different life forms and life-history traits. For a better understanding of the possible causes, we investigated the effect of population size and isolation on the genetic diversity in *falcata* populations.

2.3. Genotypic variation

As *falcata* is a clonal plant, it is also important to be aware of its genotypic diversity, which reflects the overall and spatial distribution of genetic diversity between and within populations. The genetic diversity and the formation of local genetic structure in populations of clonal species has been shown to depend on the amount of sexual reproduction by seeds and subsequent vegetative spread. Sexual reproduction is important for clonal plants because it adds new genotypes for the maintenance of genetic diversity (Widén *et al*. 1994, Honnay and Jacquemyn 2008, Silvertown 2008). Genetic diversity in populations of clonal plants is also preserved due to the accumulation of somatic mutations in buds (Antolin and Strobeck 1985, D'Amato 1997, Klekowski 1997) and by sexual reproduction through gene flow from neighbouring populations (Silvertown 2008) or from soil seed banks (Templeton and Levin 1979, Kalamees and Zobel 2002). Different species-specific traits, biological characteristics (e.g., mating system, life history traits, etc.) and habitat conditions (Hamrick *et al*. 1979, Loveless and Hamrick 1984) lead to heterogeneous spatial genetic structure at different spatial scales. The genotypic and spatial diversity of populations varies species-specifically (Honnay and Bossuyt 2005, Reisch *et al*. 2007, Honnay and Jacquemyn 2008). Moreover, the relative contribution of sexual and vegetative reproduction may vary widely within species, depending on the habitat conditions of populations, up to complete loss of sexual reproduction (Eckert 2001, Silvertown 2008). This indicates the importance of comparative studies of spatial structure and genotypic diversity in species with various life history traits, both within and among populations of

the same species growing in various habitats. Such studies would help to understand factors shaping the genotypic structure and diversity in populations of plant species with different growth forms and life history types.

3. AIMS OF THE STUDY

In the *Medicago sativa-falcata* complex, hybrids between domesticated taxa and wild plants are common, and in many regions pure *falcata* populations have disappeared or are threatened through introgressive hybridization (Small and Brookes 1984, Rufener Al Mazyad and Ammann 1999, Bleeker *et al*. 2007). As to Estonia different cultivars of *sativa* and *varia* have been introduced we excpected to see wide distribution of hybrid plants in Estonian natural populations of *falcata*. As natural populations of wild relatives of crop plants offer valuable genetic material for plant breeding, the need for preserving genetically unique natural populations is increasing (Paper **I**). Therefore it is also important to study genetic variability (Paper **II**) and reproductive biology (Paper **III**) of pure *falcata* populations, especially when these populations are threatened by habitat fragmentation and destruction. Population genetic theory predicts that in small and isolated populations the genetic diversity decreases and populations face negative effects which make them prone to extinction (Young *et al*. 1996). Therefore we expected to find a correlation between population size and the level of genetic diversity in fragmented *falcata* populations. Clonality has a strong impact on the population structure of plants and is shaped by habitat conditions (Silvertown 2008). Considering it, we expected to see that *falcata* populations consist of different genets with different sizes originated due to the local small scale vegetation disturbances and that the spatial clonal structure is influenced by ecological conditions of populations.

To clarify the status of Estonian natural *falcata* populations, the following questions are addressed:

1. How extensive is the distribution of hybrid plants among Estonian natural *falcata* populations (Paper **I**).
2. In which way has hybridization influenced two morphological characteristics (flower colour, pod shape) of plants in mixed populations (Paper **I**)?
3. Have habitat type or changes in the land use affected the abundance of hybrid plants (Paper **I**) in populations?
4. Is there any relationship between the population size, isolation distance and allozyme genetic diversity in fragmented pure populations of native *falcata* (Paper **II**)?

5. Is the geographic position of a population related to the genetic differentiation between populations (Paper **II**)?
6. How does clonal diversity and spatial distribution of genets vary within and among populations depending on the sampling scale (Paper **III**)?
7. How are the clonal structure and diversity associated with the ecological conditions and spatial heterogeneity of populations (Paper **III**)?

4. MATERIAL AND METHODS

4.1. Study species

Medicago sativa ssp. *falcata* is a perennial herbaceous plant with two possible ploidy levels - autotetraploid (2n=32) or diploid (2n=16). However, among the Estonian natural populations, so far only tetraploid *falcata* populations have been found (Kaljund and Leht 2010). The taxon is outcrossing and partially self-incompatible. *Medicago sativa* ssp. *falcata* is an insect-pollinated plant, with bumblebees (*Bombus* spp.) and solitary bees (*Megachile* spp., *Andrena* spp. and others) as the primary pollinators (Martin *et al*. 1998). Without insect pollination, the flowers are self-pollinated, which leads to increased seed abortion and inbreeding depression of the selfed progeny, presumably through the expression of lethal or deleterious alleles in the homozygous condition (Cooper *et al*. 1937, Whitehead and Davis 1954, Busbice 1968). *Medicago sativa* ssp. *falcata* reproduces sexually by seeds and vegetatively by rhizomes. Seeds of *falcata* are characterized by hard seededness. Seeds have coats that are impermeable to water due to their thickened outer walls (Bass *et al*. 1988, Bagavathiannan and van Acker 2009). Hard seededness contributes to discontinuous germination and to the formation of a persistent soil seed bank. *Medicago sativa* ssp. *falcata* spreads vegetatively by rhizomes (spreads laterally by underground stems) or by creeping roots (adventitious shoots form on horizontally growing roots) (Murray 1957, Heinrichs 1963, Lesins and Lesins 1979).

4.2. Study sites, populations and sampling

The study sites in West and North Estonia are situated in the distribution area of native *falcata*. The study summarized in Paper **I** comprised 106 populations settled on roadsides, grasslands, alvars, fallow fields and wasteland. Pure populations of *falcata* studied in Paper **II** and Paper **III** are located in North Estonia along the coast of the Gulf of Finland and in West Estonia near the town of Haapsalu and on Vormsi Island, and are settled on natural and semi-natural grasslands with calcareous light soils (Fig. 1).

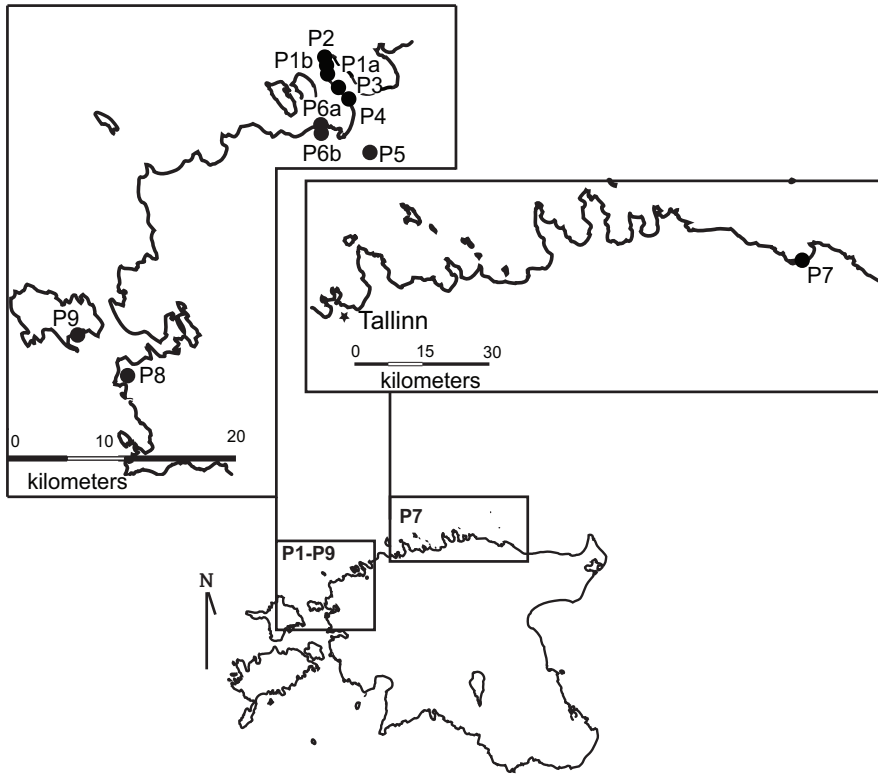

Fig. 1. Map of the 11 sampling sites for genetic diversity and genotypic structure analyses in North and West Estonia (Papers **II** and **III**).

The main study area is situated in North-West Estonia around the town of Paldiski and extends 20 km westward to the village of Kurkse. The whole region is characterised by strong human impact, with the main effects being fragmentation and habitat destruction. The Paldiski region was a closed Soviet military zone from 1940 until 1995. Because of this long period of isolation, pure populations of *falcata* have remained in the region without introgression from cultivated *sativa* or *varia*. The *falcata* populations grow in remnant grassland fragments that are separated from each other by forests, degraded habitats, buildings and roads. Populations P1$_{a,b}$, P2, P3, P4, P5, P6$_{a,b}$ (subpopulations are labelled by lower case letters) grow in the described district. Populations P1$_{a,b}$ and P2 are situated on alvars north of Paldiski, while P3 and P4 are located south of Paldiski. Populations P5 and P6$_{a,b}$ in the Kurkse region are former grasslands isolated from other ssp. *falcata* populations by unsuitable habitats such as human lodgings, roads, forests and others. Population P7 is situated directly at the coast of the Gulf of Finland in North-East Estonia on an

overgrown sand dune of a public beach. Population P8 (Paper **III**) is located on a former military airfield area near the town of Haapsalu in West Estonia. Population P9 (Paper **III**) is a former large pasture situated on the island of Vormsi in West Estonia. In the populations P5, P6 and P7, some minor contamination was noticed with only a few plants of variegated flower colour. Please note: the population numbering in the thesis differs from that in the original papers. Populations are delimited as reproductively isolated from each other through gene flow, according to possible pollination by bumblebees at distances exceeding maximum foraging ranges of *Bombus* spp. which are reported to vary from 450 m for *B. lapidarius* and *B. pascuorum* to about 760 m for *B. terrestris* (Knight *et al*. 2005). Populations with isolation distances less than pollination distances are designated as subpopulations, labelled by lower case letters ($P1_{a,b}$ and $P6_{a,b}$). Size of populations ranged from 1.5 ha to 165 ha (Paper **II**). Population size was estimated in hectares since it was impossible to count the exact number of individuals. This is because shoots of neighbouring *falcata* plants are interwoven, and genets can not be distinguished from ramets. The isolation distances between populations ranged from 1 to 170 km, whereas distances between subpopulations were 0.5-0.8 km. Population isolation is measured as an edge to edge distance to the nearest population.

Paper **I** aimed to describe how hybridization has influenced the morphology of plants by recording the two diagnostic morphological characteristics, the colour of flowers and shape of pods. Yellow flower colour, sickle-shaped pods, straight pods and corkscrew-twisted pods were assigned to the *falcata,* and variegated flowers (pale-yellow, greenish-yellow, mixed purple-yellow and brownish) and coiled pods were attributed to plants of hybrid origin. To estimate how habitat type and changes in land use may have influenced the distribution of hybrid plants, the approximate coverage percentage of *Medicago* plants in each population was estimated separately for yellow flowered *falcata* and for hybrid plants. Changes in land use due to human activity were recorded on the basis of aerial photographs from the 1950s, which were compared with the current situation as described for 67 populations during our fieldwork.

For a study of genetic diversity among the seed progeny (Paper **II**), seeds were collected as mature pods from a single branch of individual mother plants spaced at least 3-5 meters apart throughout populations

and subpopulations. From each population and subpopulation 52-89 seedlings grown from seeds of 12-15 mother plants were analysed. Seeds collected in September were maintained in a refrigerator at 2-5°C until March, then scarified with sandpaper and germinated for two days on two layers of watered filter paper in Petri dishes at 27°C in a thermostate and thereafter in a growth chamber (12 h light at 25°C: 12 h dark at 15°C) for an additional 3-4 days.

For a study of clonal diversity and structure (Paper **III**), the upper 5-7 cm parts of shoots with younger leaves were collected from all shoots (ramets) growing directly from the soil in two to three small-scale (1 m^2) plots per population from six different local populations. In order to determine the extent of clonal spread at a medium scale, a set of 35 ramets were also sampled near the nodes of 35 equal 20x20 cm grids in three 4 m^2 plots selected from the largest population P4, with a high density of *falcata* plants, from a small isolated population P6, and from a medium-sized population P1. Linear transects 30-60 m long from populations P1, P4, P6 and P8 were also analysed by sampling ramets spaced sequentially at distances of 0.7-1.5 m from each other in order to evaluate genotypic diversity at a larger spatial scale.

4.3. Electrophoretic allozyme analyses

Electrophoretic analyzes were performed as described in Papers **II** and **III**. Among nine enzymes tested in preliminary analyses, the following four isozymes proved sufficiently polymorphic and had genetically interpretable electrophoretic phenotypes with distinct allozyme bands suitable for the delimitation of allozyme-based multilocus genotypes: IDH-A (isocitrate dehydrogenase, EC 1.1.1.42), PGI-A (phosphoglucoisomerase, EC 5.3.1.9), PRX-E (peroxidase, EC 1.11.1.7) and LAP-A (leucine aminopeptidase, EC 3.4.11.1).

Electrophoretic isozyme phenotypes (hereafter zymograms) were genetically interpreted as one-banded homozygotes or multiple-banded heterozygotes with two, three or four allozyme bands and taking into account the monomeric versus dimeric structures of enzymes. Balanced and unbalanced heterozygotes were recorded by the differential staining intensity of bands on heterozygous zymograms, consistent with the autotetraploid nature of alfalfa (Quiros 1982).

4.4. Data analysis

Data for Paper **I** were analysed with the statistical software STATISTICA (StatSoft Inc. 2008). Analysis of variance combined with Tukey´s post hoc test (one-way ANOVA) was used to explain the relationships between the population characteristics (habitat type and land use changes in the studied populations) and the percentage of hybrid plants in populations. The relationship between colour of flowers and shape of pods was tested using the chi-square test.

Landscape analysis was applied to estimate changes in habitat types (Paper **I**). Land use changes in the studied populations were described by interpretation of aerial photos (large-scale ortophoto maps 1:10000) from 1951-1957 using the mapping package MapInfo Professional (MapInfo Corporation 2004).

In Paper **II**, allozymic genetic diversity among the germinated seedling progeny of populations was estimated according to the observed and expected heterozygosities H_o and H_e of Nei (1972), the mean number of alleles at the polymorphic loci analysed (A), and the effective number of alleles (A_e) using TETRAPLOIDE software (Decarli and Leinemann 2003). Values of Wright's inbreeding coefficient *F* were computed from the H_o and H_e estimates according to the equation $F=1-(H_o/H_e)$. Heterozygosity at the population level and among the seed progeny was characterized by the proportion of two, three and four-allelic heterozygotes and by the observed heterozygosity (H_o). Allele frequencies at the isozyme loci for populations were provided by TETRAPLOIDE. An UPGMA dendrogram of relationships between populations, based on the allele frequency data and Nei's standard genetic distances (Nei 1972), was constructed with DISPAN software (Ota 1993). For comparison, an UPGMA dendrogram was also constructed, using the sub-program NEIGHBOR of the PHYLIP software (Felsenstein 2009), based on the D_o genetic distances of Gregorius (1974) between the population pairs provided by TETRAPLOIDE.

For the quantitative characterization of genotypic diversity within quadrates and transects (Paper **III**), we determined genotypic richness as $R=(G-1)/(N-1)$, where G is the number of genotypes and N is the number of sampled ramets in a plot (Dorken and Eckert 2001). Each distinct multilocus genotype based on four isozyme loci is assumed

to correspond to a separate clone, and thus the genotypic richness is taken as a measure of clonal diversity. Three additional measures of clonal diversity in the study plots, the Simpson's index D, the effective number of genotypes G_e and the evenness of the effective number of genotypes E, were calculated with the use of the software GENOTYPE and GENODIVE of Meirmans and Van Tienderen (2004). D values range from zero in a population composed of a single genotype, to 1.0 when every individual sampled has different genotype. The GENOTYPE program allows a threshold value to be chosen in order to lump together very closely related genotypes that may be derived from somatic mutations. Given that isozymes are evolutionarily relatively conservative molecular markers, which frequently tend to underestimate the actual number of multilocus genotypes and clonality in species with a low effective allozyme number and polymorphic isozymes (Widén *et al*. 1994), we applied a zero threshold value for our data. To evaluate the statistical power of the four isozyme loci used for the discrimination of genotypes, the total number of possible genotypes in an autotetraploid in the four studied quadrates and transects (G_p) was calculated according to Trapnell *et al*. (2011) as

$$G_p = \prod_{i=1}^{n=4} * g_i$$

where g_i is the number of genotypes at the ith locus and n is the number of loci (n=4). The number of possible genotypes at each autotetraploid locus g_i=1/24(g)(g+1)(g+2)(g+3), where g is the number of alleles at a locus (Haldane 1948). Values of G_p were calculated for each study plot using the effective number of alleles at each isozyme locus in the respective plot, calculated with TETRAPLOIDE software (Decarli and Leinemann 2003).

5. RESULTS

5.1. Morphological variation and influences of habitat on introgressive hybridization

In the observed populations, the colour of flowers and the shape of pods varied on a large scale. The colour of flowers varied independently of the shape of pods. According to the chi-square test, yellow-flowered *falcata* plants never had coiled pods. However, plants with variegated or pale yellow flower colour may have pods characteristic of *falcata*, or coiled pods (χ^2=9.1, p=0.002). Some plants showed intermediate morphological characters compared to those of their parental taxa. These plants had greenish-yellow flower colour and pod shapes characteristic of *falcata* or *varia*. The greenish-yellow flower colour indicates first-generation hybrids between *falcata* and *varia* (Small and Brookes 1984). Comparison of hybrids with greenish-yellow flowers and yellow-flowered *falcata* revealed that plants with yellow flowers never had coiled pods, whereas hybrids had coiled pods or pods characteristic of *falcata* equally frequently (χ^2=9.3, p=0.002). Comparison of hybrids with greenish-yellow flowers with hybrids that had variegated flowers revealed that flower colour and shape of pods were not associated. Hybrid plants with variegated or greenish-yellow flower colour had coiled pods or pods characteristic of *falcata* equally frequently (χ^2=0.68, p=0.4).

Among the 106 populations studied, only 15 were pure *falcata* populations without *varia* or *sativa* plants (Fig. 2). In the remaining 91 populations, 1-90 percent of the plants showed variegated flower colour, indicating widespread but highly variable introgression. Pure populations occurred on alvars (six), on dry grasslands (four), along roadsides (three), on a fallow field (one) and one inhabited an old sandpit.

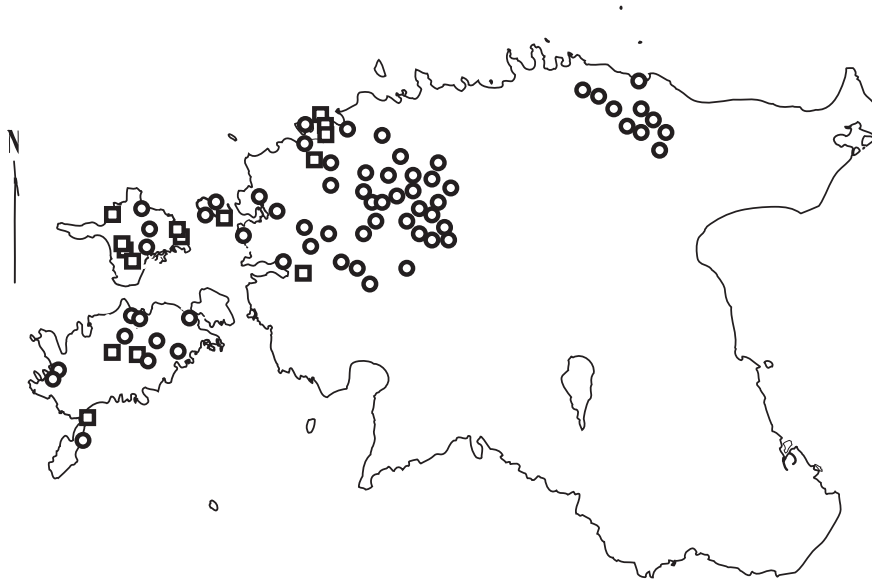

Fig. 2. Distribution map of the 106 *falcata* populations studied to evaluate the distribution of hybrid plants. Rectangles denote pure populations of *falcata* and circles denote mixed populations (Paper **I**).

Occurrence of hybrid plants did not depend on the habitat type ($F_{4,101}$=0.8, p=0.53). However, on roadsides and wastelands the percentage of hybrid plants in a population was 20-25 on average, which is higher compared to the other habitat types.

Land use changes had no influence on the abundance of hybrid plants ($F_{1,65}$=1.98, p=0.16). In populations where land use had changed, the average percentage of hybrid plants was higher, but this difference did not reach statistical significance. In populations where land use had remained unchanged, the average percentage of hybrid plants was 14%; in populations with changed land use it was on average 22%. Land use changes took place in 64% of the observed populations. According to the maps from 1951-1957, the studied populations inhabited the following habitat types: pastures, grasslands, forest, fields, shrubs, fallow fields. The observations made during fieldwork showed that only nine percent of these pastures are nowadays still used for grazing; 38% of the former pastures are now grasslands that are still mowed, and 48% are abandoned. Of the former pastures, five percent are wasteland (Fig. 3). Two former grasslands are used nowadays as grasslands. One former grassland and one former field are now abandoned sand quarries. Of

the former fields, 57% are now fallow fields and 28% are managed grasslands; wasteland, overgrown fields, and unmanaged grassland each accounted for five percent (Fig. 3).

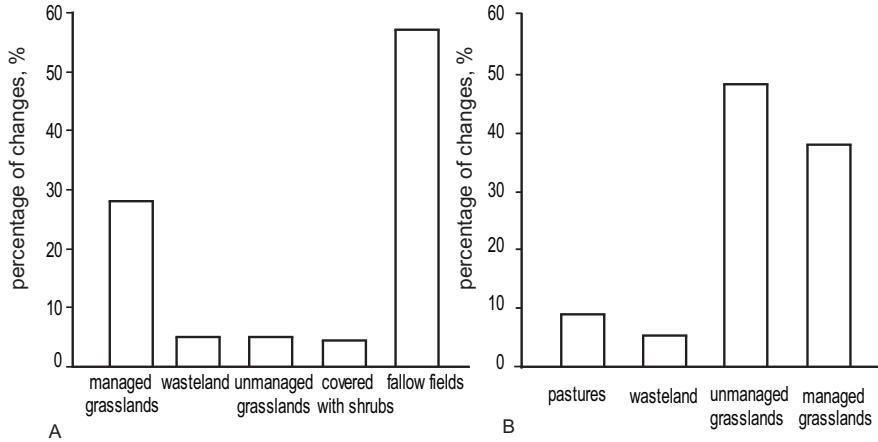

Fig. 3. Land-use changes in fields (A) and pastures (B) of 1950s. Columns show situation in 2010s (Paper **I**).

The area inhabited by two populations was in 1951-1957 overgrown with shrubs. Today the area inhabited by one of these populations is an abandoned grassland and the other is a wasteland. One former fallow field is used today as a grassland. For 36% of the populations the land use is the same as it was 60 years ago. These populations are mostly roadside populations (84%), grasslands and pastures (both 8%).

5.2. Genetic variability and differentiation of populations

All four isozymes used, IDH-A, PGI-A, PRX-E and LAP-A, were highly polymorphic, with IDH-A and PGI-A displaying variation with up to five and seven (Paper **III**) and eight (Paper **II**) allozymes, respectively, PRX-E with two frequent allozymes (Papers **II, III**), and LAP-A with five frequent allozymes (Paper **III**). The mean H_e for the isozyme loci when analysed separately varied from 0.752 for *Idh-A* and 0.823 for *Prx-E* to 0.928 for *Pgi-A*, indicating that all three loci are highly informative (Paper **II**).

Average genetic diversity measures for the seven populations are shown in Table 1 (Paper **II**). Mean number of alleles (A) for three loci ranged from 3.3 to 4.7, being highest in the smallest population P3. Effective

number of alleles (A_e) was highest in a small population P5 with A_e=2.3, whereas the largest population P4 and the smallest population P3 both had the lowest value, A_e=2. The mean Nei's panmictic genetic diversity H_e was quite high, ranging between populations from 0.795 to 0.893. Remarkably, both maximum and minimum H_e values were found for the two small populations, P3 and P5, whereas the largest population P4 has a H_e=0.808 close to that of the smallest population P3 (H_e=0.795). Regression analysis showed no significant correlations between H_e (p=0.1, R=0.3), A (p=0.5, R=0.4), A_e (p=0.7, R=0.2) or F (p=0.4, R=0.4), and population size. Regression analysis also revealed no significant correlation between H_e (p=0.5, R=0.4), A (p=0.9, R=0.03), A_e (p=0.3, R=0.5) or F (p=0.8, R=0.09), and the population isolation distance.

Table 1. Population characteristics and mean population genetic diversity measures for three polymorphic loci in nine populations and subpopulations of *falcata*. Standard deviations are given in the parentheses. S - population size (area, in ha), I - isolation by the distance to the nearest population, km, N - number of individuals analysed, A - mean number of alleles for three loci, A_e - effective number of alleles, H_o - observed heterozygosity, H_e - expected heterozygosity, F - Wright´s inbreeding coefficient (Paper **II**).

Population	S, (ha)	I, (km)	N	A	A_e	H_o	H_e	F
P1a	20	0.8	86	3.7	2.1	0.774 (0.14)	0.828 (0.11)	0.065 (0.11)
P1b	15	0.8	54	3.7	2.1	0.827(0.08)	0.840 (0.10)	0.015 (0.03)
P2	15	1.5	63	4	2	0.819 (0.02)	0.840 (0.02)	0.025 (0.01)
P3	1.5	3	69	4.7	2	0.794 (0.08)	0.795 (0.14)	0.002 (0.10)
P4	165	3	52	4	2	0.804 (0.12)	0.808 (0.12)	0.006 (0.01)
P5	4	1	62	3.3	2.3	0.803 (0.12)	0.893 (0.06)	0.103 (0.07)
P6a	1	0.5	68	3.3	2.1	0.838 (0.06)	0.838 (0.10)	0.001 (0.08)
P6b	1	0.5	73	3.7	2.1	0.763 (0.19)	0.843 (0.11)	0.095 (0.11)
P7	5	4	89	3.7	2.1	0.722 (0.19)	0.831 (0.19)	0.136 (0.16)

The seed progeny of five populations showed prevalent out-crossing ($F \leq$ 0.1), indicating that this is derived from nearly random bumblebee-mediated mating between the adult mother plants. Only population P7 had a slightly higher *F*, showing about 14 percent inbred progeny.

Data about the distribution of allele frequencies among three polymorphic isozyme loci in the studied populations (Table 2) show that only two of five *Idh-A* alleles, A3 and A4, are frequent in all populations. Alleles A1

and A2 were detected in only one or a few individuals in populations P1a, P1b, P2, P3, P4, and P6, and were absent in populations P5, P6b and P7. Likewise, only three of eight *Pgi-A* alleles, A4, A5 and A7, contribute most to the genetic diversity and its variation. Allele A8 was detected only in the farthest and most isolated Kunda population P7. Both alleles of *Prx-E* are frequent in all populations and contribute significantly to the genetic diversity.

Table 2. Allele frequencies at three polymorphic loci in nine populations and subpopulations of *falcata* (Paper **II**).

		Populations								
Locus	Allele	P1a	P1b	P2	P3	P4	P5	P6a	P6b	P7
Idh-A	1	0.015	0.014	0.005	0.043	0.037	0	0.007	0	0
	2	0.010	0	0	0.012	0.037	0	0.007	0	0
	3	0.730	0.707	0.641	0.780	0.684	0.593	0.711	0.728	0.688
	4	0.173	0.236	0.293	0.128	0.199	0.352	0.197	0.211	0.258
	5	0.071	0.043	0.060	0.037	0.044	0.056	0.079	0.050	0.053
Pgi-A	1	0	0	0.004	0.004	0	0	0	0	0
	2	0	0.048	0.032	0.022	0.043	0.064	0.011	0.010	0.014
	3	0.013	0.005	0.020	0.022	0.032	0	0.008	0	0
	4	0.174	0.221	0.067	0.127	0.106	0.360	0.462	0.372	0.164
	5	0.482	0.471	0.282	0.246	0.346	0.216	0.163	0.156	0.333
	6	0	0	0	0.011	0	0.004	0.008	0.007	0.014
	7	0.330	0.255	0.595	0.569	0.473	0.356	0.348	0.455	0.460
	8	0	0	0	0	0	0	0	0	0.014
Prx-E	1	0.385	0.375	0.375	0.445	0.270	0.415	0.381	0.473	0.323
	2	0.615	0.625	0.625	0.555	0.730	0.585	0.619	0.527	0.677

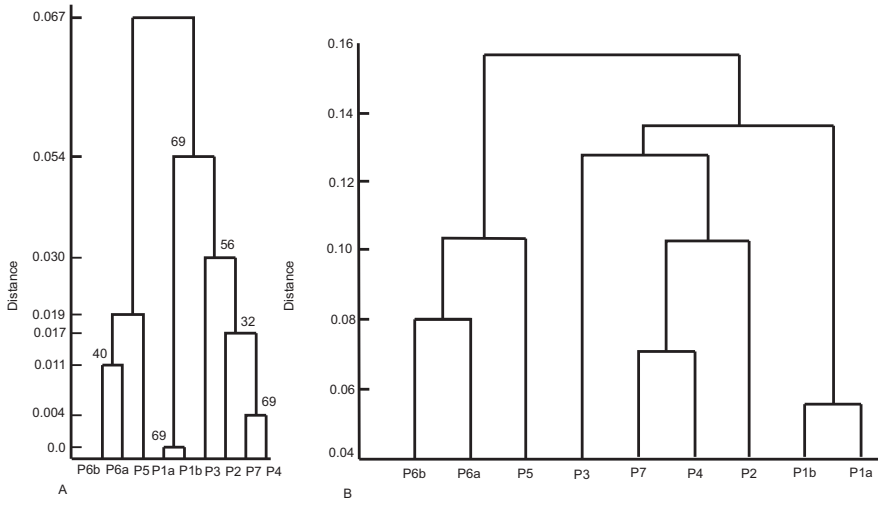

Fig. 4. UPGMA dendrograms illustrating genetic relationships in nine populations and subpopulations of *falcata*, based on Nei's standard genetic distances (A) and on genetic distances of Gregorius, D_o (B). Numbers at nodes show bootstrap values (1.000 replicates) (Paper **II**).

UPGMA cluster analysis based (Fig. 4) on Nei´s standard genetic matrix and D_o genetic distance matrix between populations revealed clear genetic differentiation into two major groups. One group includes nearby populations $P1_a$, $P1_b$, P2, P3 and P4 sited in the same Paldiski region, but also population P7 from the very distant Kunda region. The Paldiski populations $P1_a$ and $P1_b$ form a separate branch linked to populations P2 and P4 of the same region. Populations P5, $P6_a$ and $P6_b$, forming a separate group, are sited in the adjacent Kurkse region, about 15-20 km away from the Paldiski populations. Thus, populations of the Kurkse region and populations of the Paldiski region appear in two separate clusters, indicating geographic structuring in this region. However, the remote Kunda population P7 was spaced within the Paldiski group populations, with highest genetic similarity to P4, indicating a general lack of correspondence between genetic differentiation and geographical distance.

5.3. Genotypic diversity and spatial structure at different scales

The data on the genotypic diversity measures at three spatial scales are summarised in Table 3. The number of possible genotypes was always much higher than the number of genotypes found (Table 3), indicating that distinct MLGs detected may be attributed to different genets.

Table 3. Summary of genotypic diversity parameters for the $1m^2$ and $4m^2$ plots and transects in six populations of *falcata*. P - population, Q1 - 1 m^2 quadrate, Q4 - 4 m^2 quadrate, T - transect, N - number of ramets genotyped, G - number of multilocus genotypes (genets) detected, R=G-1/N-1 - genotypic richness, G_e - effective number of genotypes, E - the evenness index, D_s - Simpson's index of genotypic diversity, G_p - a total number of possible genotypes across loci, based on the effective allele number at each isozyme locus in a sample (Paper **III**).

P; Q; T	N	G	R	G_e	E	D_s	G_p
P1							
Q1a	14	2	0.08	1.15	0.58	0.14	141
Q1b	27	6	0.19	4.26	0.71	0.79	172
Q4a	35	11	0.29	10.5	0.7	0.93	1734
Q4b	35	28	0.79	21.5	0.77	0.98	555
Q4c	35	27	0.76	23.1	0.86	0.98	5036
T1	35	35	1	35	1	1	4385
T2	40	39	0.97	38	0.98	1	11670
P4							
Q1a	36	2	0.03	1.99	1	0.51	130
Q1b	34	30	0.88	26.3	0.88	0.99	3369
Q1c	38	2	0.03	1.11	0.56	0.1	47
Q4a	35	32	0.91	29.8	0.93	0.99	5737
Q4b	35	17	0.47	8.22	0.48	0.9	1119
Q4c	35	10	0.26	4.59	0.46	0.81	1944
T1	40	40	1	40	1	1	5810
T2	50	40	0.8	29.1	0.73	0.99	1571
P6							
Q1a	14	1	0	1	1	0	54
Q1b	31	1	0	1	1	0	1677
Q4a	32	7	0.19	3.32	0.47	0.72	837
Q4b	35	7	0.18	2.72	0.39	0.65	724
Q4c	34	1	0	1	1	0	206
T1	59	33	0.55	21.9	0.66	0.97	1651
P7							
Q1a	25	7	0.25	2.78	0.4	0.67	624
Q1b	30	12	0.38	7.38	0.61	0.89	4579
P8							
Q1a	23	5	0.18	1.77	0.35	0.45	104
Q1b	23	9	0.36	4.68	0.52	0.82	242
Q1c	34	12	0.33	4.21	0.36	0.79	728
T1	38	37	0.97	36.1	0.98	1	1670
P9							
Q1a	56	11	0.18	5.03	0.46	0.82	408
Q1b	34	13	0.36	9.17	0.71	0.92	4434

5.4. Genotypic diversity and spatial structure within 1 m² small-scale quadrates

A total of 112 multilocus genotypes were detected from all the 419 ramets sampled in 1 m² plots, i.e. 27% of ramets had different MLGs. Only one shared MLG was found in two geographically distant populations P2 and P5. Otherwise, each population displayed a unique set of MLGs. The indexes of genotypic diversity were highly variable among populations and between quadrates within populations (Table 3). Genotypic richness (R) varied remarkably among the 14 analysed plots, ranging from 0 to 0.88. The greatest range of variation was found in P4, where in plot Q1b, genotypic richness was 0.88, but in plots Q1a and Q1c it was only 0.03.

Most of the 1m² sites displayed highly variable spatial structure: the number of ramets in the fourteen quadrates varied from 14 to 56. The spatial structure of MLGs in a selected set of six 1 m² quadrates of three populations is shown in Fig. 5. These plots illustrate the extreme cases of variation among and within populations. Q1a of P9 comprised 56 ramets belonging to 11 MLGs (Table 3), with the number of ramets in a genet ranging from 1 to 15 (Fig. 5). Q1b of P9 contained 34 ramets of 13 MLGs (Table 3), but the number of ramets in a genet ranged from 1 to only 7. The maximum distance between the farthest ramets in a genet of the two quadrates was 0.75 and 0.4 m, respectively. These distances reflect maximum genet sizes. P6 was exceptional because the two quadrates examined (Q1a, Q1b) were monoclonal, consisting of two different genotypes with 14 and 31 ramets, respectively. The maximum genet size in the two quadrates of P6 was about 1 m.

P4 showed remarkably extreme variation between the two 1m² plots shown in Fig. 5. Q1a of P4 had only two different genotypes. The maximum genet size in Q1a and Q1b was 1 m and 0.15 m, respectively. The number of ramets in the two genets of Q1a was almost equal: 17 and 19 ramets. In a sharp contrast, in Q1b of P4 nearly all ramets had different genotypes. Only three genotypes in Q1b revealed clumped spacing of nearby growing ramets formed through clonal growth, while the remaining ramets belong to unique genotypes that have not yet propagated vegetatively (Fig. 5). This indicates that a local disturbance in Q1b, accompanied by increased sexual reproduction from seeds has occurred. We suppose that unique MLGs reflect genets derived through

sexual reproduction from seeds, whereas adjacent ramets of 2-4 identical MLGs belong to small genets that have recently started to spread clonally.

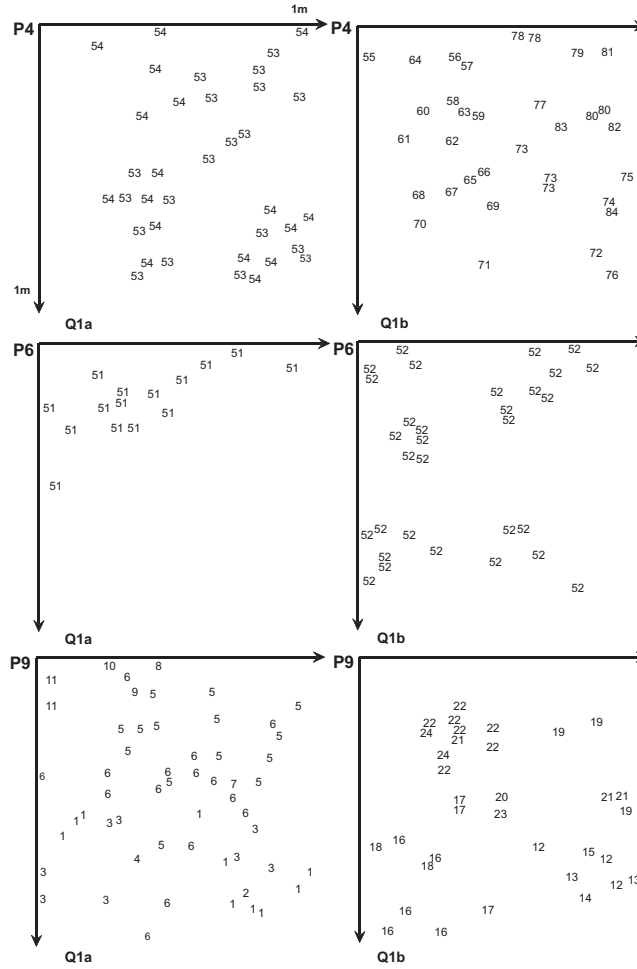

Fig. 5. Spatial distribution of multilocus genotypes in 1 m^2 quadrates (Q1a, Q1b) with all ramets sampled and mapped. Numbers in quadrates mark different multilocus genotypes (MLGs). Studied populations are marked as following: P4 (dry grassland), P6 (abandoned grassland), P9 (abandoned pasture).

5.5. Genotypic diversity and spatial structure within 4 m² medium-scale quadrates

In each quadrate, one ramet each from 35 equal subplots was analysed. A total of 144 MLGs were detected among 311 ramets in nine quadrates of three populations, i.e. 46% of ramets had different MLGs. None of

the MLGs was found in more than one population, and all quadrates had unique multilocus genotypes. The indexes of genotypic diversity were variable both among and within P1, P4 and P6 (Table 3). R varied notably among the three populations and among the nine 4 m^2 plots, ranging from 0 up to 0.91.

The 4 m^2 quadrates showed large variations also in the spatial structure and sizes of genets (Fig. 6). Clonality was the highest in P6. In two quadrates, Q4a and Q4c of P6, the genotypes were spatially aggregated and the maximum genet size was 2 m. In Q4a of P6 the number of ramets per genet varied from 1 to 16, and one genotype was dominating, while in Q4c only one genotype with 34 ramets was found. P4 showed contrasting results, with lowest clonality in Q4a and highest in Q4c. In Q4a of P4 the maximum genet size was 0.3 m and the number of ramets in a genet was 1-2. In Q4c the maximum genet size was 1.68 m, the number of ramets in a genet ranged from 1 to 11, and two genets, 177 and 180, were dominant. In Q4a and Q4b of P1 the maximum genet size was 1.15 m and the maximum number of ramets per genet was the lowest, ranging up to 4-5.

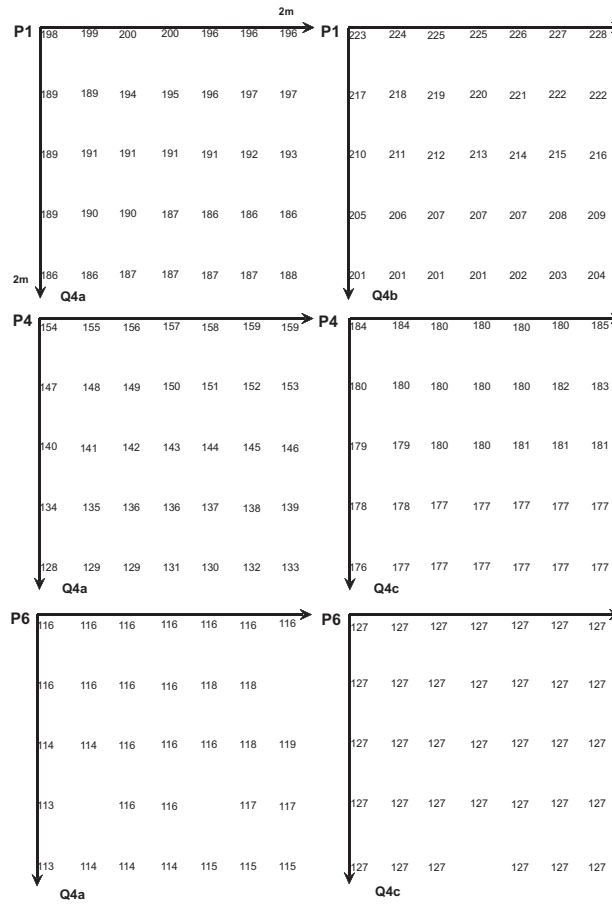

Fig. 6. Spatial distribution of multilocus genotypes in 4 m^2 quadrates (Q4a, Q4b, Q4c). Numbers in quadrates mark different multilocus genotypes (MLGs). Empty places represent missing ramets. Studied populations are marked as following: P1 (alvar), P4 (dry grassland), P6 (abandoned grassland).

5.6. Genotypic diversity and spatial structure within large-scale transects

The linear transects displayed higher genotypic diversity than quadrates (Table 3). The number of MLGs detected reached 223 among the total 262 ramets analysed. The share of different MLGs has thus increased from 27% in 1 m^2 quadrates to 85% in transects. Only one shared genotype was found: in P4 and P1 two individuals had the same MLG. Clonality along the 60-m-long linear transect of P6 was highest (R=0.55), whereas transect T1 of P4 and transects T1 and T2 of P1 revealed no clonality

(R=1.0), despite the fact that in P4 and P1 considerable clonality was observed in most small- and medium-scale quadrates. This shows that studies at different and appropriate spatial scales are needed in order to obtain reliable information about the extent and variation of clonality. In a transect of P6, 18 out of 59 analysed ramets had unique genotypes and the maximum genet size was about 6 m. In P4, transect T2 also revealed moderate clonality (R=0.80), in contrast to transect T1, where all sampled ramets had unique genotypes. These data show that extensive large-scale sampling of adult plants of a clonal species from ecologically different sites of a large population such as P4 is needed to obtain reliable data on the population-level genotypic diversity

6. DISCUSSION

6.1. Variation in diagnostic morphological traits

Traditionally, flower colour and pod shape characters have been used to distinguish hybrid plants in the *M. sativa-falcata* complex. Even so, sometimes it is confusing to distinguish hybrid plants on the basis of morphological characters. For example, two Estonian *varia* cultivars bred for pasture have pale yellow flowers and their pods may vary from sickle-shaped to spirally coiled (Bender 2006). Therefore, yellow-flowered *varia* has occasionally been misidentified as *falcata* in Estonia.

There is a continuum of variation between *falcata*, *sativa* and *varia*. Our study also showed that the colour of flowers and the shape of pods varied widely in the observed populations. It has been reported (Small and Brookes 1984) that in hybrid populations, odd character associations, like yellow-flowered plants with coiled fruits, are frequent in some parts of the world. We found that yellow-flowered *falcata* plants in Estonia never had highly coiled pods, but their pods were quite often corkscrew-twisted. Plants with variegated flower colour may have pods characteristic of *falcata*, or even coiled pods characteristic of *sativa*.

However, no specific molecular markers have yet been found to distinguish cultivated and wild forms, although the populations of wild and cultivated plants have been shown to be genetically differentiated from each other by allele frequencies (Jenczewski 1998, Jenczewski *et al.* 1999a, 1999b, Muller *et al.* 2001, Greene *et al.* 2008). Because of the large overlapping of morphological characteristics and molecular data in the *M. sativa-falcata* complex, both selected molecular markers and morphological characters are needed to study introgressive hybridization in more detail.

6.2. Distribution of hybrids in populations depending on the habitat type and changes in land use

Medicago sativa ssp. *falcata* has been intensively used for improving the winter hardiness and grazing tolerance of *varia* cultivars. Therefore differences in the requirements for habitat and environmental conditions

between *falcata* and *varia* have diminished, and domesticated plants of *varia* are able to survive and spread under natural conditions. This is very evident in Canada where roadside feral populations of escaped cultivars with high parentage from *falcata* are very viable (Bagavathiannan and Van Acker 2009, Bagavathiannan *et al*. 2010). These populations seem to be governed by natural selection for winter survival and clonal growth, traits which are presumably supporting the persistence of roadside hybrid populations (Bagavathiannan and Van Acker, 2009, Bagavathiannan *et al*. 2010). In Estonia, traits of *falcata* integrated in hybrids may similarly support the establishment of hybrid plants in natural populations on alvars and dry grasslands in addition to roadside habitats, as only six populations growing spatially isolated from cultivated forms appeared to be pure *falcata* populations.

It has been shown that gene flow by pollen from cultivated *sativa* and *varia* fields may cover long distances, from 450 m up to 760 m, depending on the pollinating *Bombus* species (Knight *et al.* 2005). Furthermore, gene flow via seeds mediated by humans can also be significant and may increase gene exchange between natural and cultivated populations (Muller *et al*. 2001). Therefore it can be assumed that the five pure populations on the roadsides, in the fallow field and sand quarry are probably recently established by initial seedling recruitment from *falcata* seeds. Hybrid plants are still missing in those populations.

Human activities often give rise to ruderal habitats that can easily be colonized by taxa with high dispersal ability. In disturbed habitats, formerly separated taxa may come into contact since human activities have destroyed ecological and spatial barriers (De Wet and Harlan 1975, Levin *et al*. 1996). In many cases it is not known if *falcata* and *varia* are growing on pastures and grasslands naturally or have been sown there during the period of intensified agricultural practices from 1950 to 1990. One can claim that cultivation of grasslands and pastures has historically contributed to the better distribution of hybrid plants, making co-occurrence of the two taxa possible. However, we did not find any significant difference in the occurrence of hybrid plants on abandoned land compared to sites where the land use had remained the same, although escaped cultivars obviously have more favourable conditions for spreading along roadsides. It is evident that *varia* has adopted traits of *falcata* that support the persistence of hybrid plants in man-made as well as in natural habitats.

6.3. Genetic variability and population differentiation

The results revealed no significant association between genetic diversity, population size, and isolation distance. This means that the fragmentation of the initially large grasslands of North Estonia into smaller and isolated patches through anthropogenic habitat destruction during the last century has not yet caused the expected reduction of genetic diversity.

There are contradictory reports in literature about the effect on genetic diversity of habitat fragmentation into smaller population patches (Young *et al*. 1996). Consistent with theoretical predictions, many studies have shown that fragmentation has a strong impact on the genetic diversity, with smaller populations showing a loss of genetic diversity (reviewed by Ellstrand and Elam, 1993, Leimu *et al*. 2006, Honnay and Jacquemyn 2007, Aguilar *et al*. 2008). However, and consistent with our study, there are also many papers reporting no significant correlation between H_e and population size for many other herbaceous perennials (Waldmann and Andersson 1998, Gustaffson 2000, Mateu-Andres and Segarra-Moragues 2000, Jacquemyn *et al*. 2004, Pluess and Stöcklin 2004, Leimu and Mutikainen 2005, Bachmann and Hensen 2007, Honnay *et al*. 2007, Kuss *et al*. 2008).

Several studies have found no correlation between population size and the genetic diversity measure H_e, but that population size appears to be positively correlated with the proportion of polymorphic loci and the mean observed number of alleles (Van Treuren *et al*. 1991, Raijmann *et al*. 1994, Young *et al*. 1999, Van Rossum *et al*. 2002). Some studies have shown that the number of alleles is more sensitive to the reduction of population size than the level of heterozygosity. It is remarkable that we found no correlation between population size and any of the three estimated measures of allozyme genetic diversity A, A_e or H_e. Different alternative explanations for the maintenance of genetic diversity in small habitat fragments are discussed in literature reviews (Leimu *et al*. 2006, Honnay and Jacquemyn, 2007, Aguilar *et al*. 2008, Vranckx *et al*. 2011). One possible explanation is that not enough time has elapsed since the beginning of habitat fragmentation to cause a notable decrease of genetic diversity. This is most evident for long-living trees (e.g., Young *et al*. 1993) and herbaceous perennials (e.g., Rosquist and Prentice 2000), as they respond slowly to a decline in changing habitat conditions (Ellstrand and Elam 1993). However, a loss of genetic diversity has been observed

in small forest fragments of several long-lived trees, e.g, for *Halocarpus bidwillii* (Billington 1991), *Eucalypyus albens* (Prober and Brown 1994), *Pithecellobium elegans* (Hall *et al*. 1996), and *Fagus sylvatica* (Jump and Peňuelas 2006), and for other tree species. And contrary to theoretical expectations, no loss of genetic diversity could be found in small and isolated populations of several annuals (Evans *et al*. 2000, Geimler and Dobeš 2000, Podolsky 2001, Spigler *et al*. 2010). As *falcata* is perennial, it is plausible to assume that insufficient time has passed to express the consequences of genetic drift and inbreeding on fragmented population patches of North Estonia. However, negative responses to habitat fragmentation for woody plants are usually more visible in offspring, while adult trees evidence genetic extinction debt and reflect the historical genetic diversity (Vranckx *et al*. 2011). Therefore, other characteristics of *falcata* besides perennial life form can contribute to the maintenance of high genetic diversity.

Our results showing high levels of heterozygosity among the viable seedling progeny indicate a strong selection acting against inbred homozygous offspring, which is beneficial for population survival. As shown in literature, high seed abortion through early-acting inbreeding depression is characteristic of *sativa* (e.g., Cooper *et al*. 1937, Busbice 1968 and others.) A high level of early inbreeding depression causes the abortion of homozygous genotypes formed by selfing. Abortion of self-fertilized embryos, also described in literature as early-acting inbreeding depression or late-acting incompatibility, is widely distributed among outcrossing plants of various taxonomic groups (reviews: Seavey and Bawa 1986¸ Charlesworth and Charlesworth 1987). Early-acting inbreeding depression is considered an effective evolutionary mechanism for purging genetic load caused by deleterious alleles exposed to selection in selfed, homozygous seed progeny (Charlesworth and Charlesworth 1987, Charlesworth 1989). Therefore it is a plausible mechanism to explain the observed significant genetic diversity and no increase in the F values, except in population P7, in a small population of *falcata*. The genetic diversity among the seed progeny is derived from bumble-bee mediated crosses between reproductive adult plants and thus reflects the genetic diversity existing in a wild population.

Another important factor for preserving genetic diversity is obviously the persistent seed bank. The yearly new contribution to the seed bank

provides continuity to the genetic memory and is an important factor for the maintenance of genetic diversity by acting against the genetic erosion in small and isolated populations. A paper of Honnay *et al.* (2008) reviews studies providing evidence that a persistent seed bank may mitigate the consequences of habitat fragmentation and protect small and isolated plant populations from genetic drift and local extinction. Alfalfa has a high percentage of seeds with hard coats impermeable to water, allowing them to remain dormant for years and to contribute to the formation of a persistent seed bank (e.g., Acharya *et al.* 1999). We found that six months after collecting and maintaining in a refrigerator, scarification was still needed to germinate the seeds of *falcata*, indicating their potential to form a durable seed bank.

Finally, the autotetraploid nature of ssp. *falcata* may additionally contribute to maintaining high levels of genetic diversity. Autotetraploids have higher level of individual heterozygosity with up to four different alleles, instead of two in diploids. Isozyme studies have shown that autopolyploids display significantly higher levels of heterozygosity than their diploid relatives (e.g., Lumaret 1982, Soltis and Soltis 1989, Brown and Young 2000, Hardy and Vekemans 2001, and others). In addition, autopolyploidy slows down the loss of heterozygosity at neutral and nearly neutral loci through inbreeding in small populations, simultaneously allowing the elimination of recessive lethal and highly deleterious alleles from the seed progeny through early-acting inbreeding depression, as shown for autotetraploid alfalfa (Cooper *et al.* 1937, Whitehead and Davis 1954, Busbice 1968). However, polyploidy alone, without inherent early-acting inbreeding depression, may be insufficient for preventing genetic erosion, as shown for fragmented populations of a tetraploid pea *Swainsona recta* (Buza *et al.* 2000) and for isolated populations of different sizes of the autotetraploid Pyrenean endemic *Delphinium montaneum* (Lopez-Pujol *et al.* 2007).

A notable result is that genetic differentiation of populations into the two major groups appears to be associated with soil conditions. The Kurkse populations P5 and P6$_{a,b}$ of the first group are abandoned pastures situated on nutritious soils with deeper humus depth, over 20 cm. The Paldiski and Kunda populations of the second major group are remnant fragments of calcareous grasslands or are growing on nutrient poor sandy soils. At the same time, there was no general correspondence between the geographical distances and genetic differentiation of populations.

In summary, the autotetraploid nature, combined with early-acting inbreeding depression and yearly submission of highly heterozygous seed progeny to the seed bank are plausible factors contributing to the maintenance of high genetic diversity in small and isolated populations of *falcata* in fragmented Estonian alvars. The data on the maintenance of high allozyme genetic diversity in small and isolated grassland fragments of *falcata* support the view that perennial grassland species respond slowly to habitat loss and fragmentation by exhibiting extinction debt (Helm *et al*. 2006, Kuussaari *et al*. 2009).

6.4. Genotypic variability and spatial structure of populations in relation to habitat conditions

We found that genotypic diversity and spatial structure vary substantially between and within populations, even among neighbouring 1 m^2 and 4 m^2 plots of *falcata*. Most authors have found high levels of genetic diversity in clonal species at the species level. Thus, some have recorded quite a large variation in the genotypic diversity between neighbouring populations of the same clonal species, for example in populations of *Festuca rubra* (Rhebergen *et al*. 1988) and *Fragaria virginiana* (Wilk *et al*. 2009). The other extreme can be that no genotypic allozyme and RAPD variation is detected within and among populations, like the invasive clonal species *Fallopia japonica* throughout Europe (Hollingsworth and Bailey 2000, Mandak *et al*. 2005, Krebs *et al*. 2009). It has been suggested that the spatial genotypic structure and clonal diversity of populations largely depend on sexual reproduction and vegetative growth, subjected to the ecological conditions in the population. An important ecological factor for the successful sexual reproduction is the availability of suitable sites for seedling recruitment from the seed pool, which may vary both between and within populations, depending on disturbances (Eriksson 1989, Eriksson and Eriksson 1997, Honnay and Bossuyt 2005).

Among the studied *falcata* populations, vegetation density seems to be another important ecological factor shaping the relative contribution of sexual reproduction from seeds, and subsequent vegetative spread of genets. Population P6 of an abandoned small grassland differs from other populations due to its dense vegetation with dominating high grasses and thick litter layer. These conditions obviously suppress sexual reproduction through seedling recruitment. Our data suggest that such

habitat is associated with the existence of monoclonal quadrates with low or no clonal diversity in P6. We suppose that the most likely explanation for the locally reduced genotypic richness in P6 is that clonal competition has eliminated less fit genotypes and has favoured the vegetative spread of genotypes adapted to the dense vegetation. Maturing and aging of the population has presumably favoured selection of locally superior clones with enhanced vegetative growth. A decline in the genotypic richness and a concomitant increase in clonal reproduction, together with the aging of the population and the establishment of dense vegetation cover, has been reported in several studies of different species (Piquot *et al.* 1998, Silvertown 2008, and references therein). The presence of monoclonal quadrates shows that seedling recruitment may locally be even completely suppressed in sites with a dense vegetation cover, and may occur only in places that have been subjected to occasional micro-disturbances. It has been shown that sexual reproduction by seedling recruitment is largely or totally suppressed in established grasslands covered with a thick litter layer in spring and with a dense undisturbed vegetation of adult herbs in summer (Aspinwall and Christian 1992, Xiong and Nilsson 1999, Vandepitte *et al.* 2010). Evidently, local disturbances are needed to provide open microsites for safe seedling recruitment in places with a thick litter layer and dense vegetation, characteristic of P6, P9 and some regions of P1, P4 and P8, where quadrates with high or moderate genotypic richness were found. Similarly, P7 is a small population situated on a sandy beach, where trampling by people may have caused small local disturbances associated with a moderate genotypic diversity in $1m^2$ quadrates. The results obtained indicate that there is a clear small-scale spatial structure in the distribution of clonal diversity, in which neighbouring ramets form clumps of closely spaced clonemates and different dominating MLGs in quadrates can be observed.

Populations established to a great extent through sexual reproduction from seeds are expected to consist of a large number of randomly distributed genotypes (Ellstrand and Roose 1987). Indeed, the genotypic richness reached 0.55 in a large linear transect extending diagonally through the whole of P6, indicating input of new genets by sexual reproduction at local micro-sites in this population. Similarly, $1m^2$ and $4m^2$ plots with a high level of genotypic richness were found in populations P4 and P1, evidencing a significant level of sexual reproduction in some small sites within these populations. In this respect, large differences among the

three 1m^2 plots of P4 are of special interest, because they were sampled in a region with a uniformly dense cover of *falcata* plants.

A notable outcome is that the genotypic diversity measures decline with sampling scale, from a maximum in 1m^2 quadrates up to no evidence of clonality or only minor clonality in several long linear transects. A similar loss of evidence of clonality at large sampling scales was also noted by Widén *et al*. (1994). These data emphasise the importance of performing analyses at different and appropriate spatial scales, in order to obtain reliable information about the extent of clonality and its variation within populations. Another important finding is that the two transects sampled from different places in P4, and which differ in vegetation density, provided quite different results on the genotypic richness and evenness. This shows that extensive large-scale sampling of adult plants of a clonal species from ecologically different sites of a large population is needed in order to obtain reliable data on the population-level genotypic diversity.

We hypothesise that the soil seed bank may be the main source of sexual reproduction in populations of *falcata* following local disturbances. *Medicago sativa* ssp. *falcata* has hard seeds with a water impermeable coat, thus favouring the formation of a persistent soil seed bank and providing the opportunity for the maintenance of high genetic diversity through sexual reproduction even in small and fragmented populations (Bass *et al*. 1988). However, we also observed consistently high yearly seed production in the study populations, indicating a possible contribution to clonal diversity also from the annual seed rain in places of sparse vegetation, or following occasional recent local disturbances of different origin and extent in populations with dense vegetation. Bagavathiannan *et al*. (2011) demonstrated that feral alfalfa (*sativa*) is able to establish successfully from seeds sown to grass swards, but the degree of establishment depended on the stand density and disturbance. The same seems to apply also to ssp. *falcata* in its native grassland populations.

6.5. Future prospects

The results of the study showed that pure populations of *falcata* in Estonia have high genetic variability and genotypic richness. The next problem that needs to be studied is the comparison of genetic variability and

structure between older source populations and nearby newly established sites, since spatial processes are important for determining the structure and dynamics of populations and communities. Spreading of a plant population by colonising spare space depends on many factors, first of all on the dispersal ability by seeds. Crucial for the establishment by seeds are the requirements for germination conditions and the existing vegetation cover in the area to be colonised. Dispersal determines the structure and dynamics of populations and affects genetic structure within and among the founded populations. In newly established sites we would expect to find a decrease in heterozygosity, the number of alleles per locus, and the genetic divergence between source populations and newly established sites.

We established that, in older populations with dense vegetation cover including competitive grasses, some genotypes have become locally more abundant, even up to the formation of monoclonal plots. We suggested that this may be caused by competition between *falcata* genets and neighbouring plants. Population genetic theory suggests that there should be a positive relationship between individual multilocus heterozygosity and fitness, due to heterozygous advantage. Numerous studies have indeed found such correlations for many species, caused by adaptation to local environments. However, many other studies have received contradictory results, indicating a need for further studies in order to understand the reasons for the discrepancies. Therefore, we are going to test if there is a positive correlation between more abundant larger genets and higher levels of heterozygosity, on the example of a clonal species.

7. CONCLUSIONS

The thesis discusses an important issue about hybridisation between domesticated taxa and their wild relatives, on the example of the *Medicago sativa-falcata* complex in Estonia. Our study showed that *sativa* or *varia* plants are present in most Estonian *falcata* populations, leading to the loss of native, genetically pure *falcata* populations. As the remaining pure *falcata* populations are mostly small and isolated, the influences of fragmentation on genetic diversity were also under study. Our study is the first to describe genetic diversity in fragmented natural populations of *falcata* in relation to the population size and biological characteristics of *falcata*, as well as the clonal diversity and spatial structure in relation to the habitat conditions of *falcata* populations. The results showed that the remaining pure small and isolated *falcata* populations still have very high genetic diversity in seed progeny and display a wide variation in the genotypic richness and spatial clonal structure among adult plants. We can conclude that the sexual recruitment of new genotypes reveals the importance of the seed bank or annual seed rain for the maintenance of genetic diversity in these populations.

From the results of present thesis the following conclusions can be drawn:

- The results indicate ongoing and variable introgressive hybridization from introduced cultivars of *sativa* and *varia* in Estonian natural populations of *falcata*.
- Morphological characteristics distinguishing between *varia* and *falcata* have become more diverse in hybrid plants, due to introgressive hybridization in mixed populations, extending the results of related studies. Pure populations of *falcata* contain only individuals with yellow flower colour and sickle-shaped pods, while hybrid plants in mixed populations include individuals with pod shape and flower colour characteristic of *falcata*, *sativa* or *varia*.
- There is a trend that most favourable habitats for the growth and dispersal of hybrid lucerne plants are disturbed man-made habitats, such as roadsides, fallow fields and wasteland were the percent of hybrid plants was the highest.
- We did not find correlation between population size, isolation degree and genetic diversity. Specific biological features of

falcata, like early-acting inbreeding depression, autotetraploidy, perennial life form and a continuously renewing seed bank, are proposed as crucial factors that support the persistence of genetic diversity in its currently small and isolated populations, which have been formed as a result of habitat destruction and fragmentation.

- The indexes of clonality vary widely within and among the *falcata* populations, depending on the sampling scale and decreasing from a maximum in small $1 m^2$ quadrates to no apparent clonality or only moderate clonality in six long linear transects. This indicates the importance of selecting appropriate and different sampling scale to characterise local variation in clonality in response to environmental heterogeneity within populations, and the overall clonality between populations.
- *Medicago sativa* ssp. *falcata* populations display wide variation in genotypic richness and spatial clonal structure. This is associated with variable environmental conditions and the different ecological history of populations. The degree of establishment sexually from seeds of *falcata* in natural grasslands depends on the vegetation cover and disturbances. Consistent with studies of other plant species, local disturbance events in stable populations with dense vegetation provide opportunities for occasional sexual reproduction from the seed bank and annual seed rain. But in stable aging populations with dense vegetation cover, reproduction from seeds is locally suppressed and only some successful genets with wide distribution become dominating.
- Pure *falcata* populations on calcareous grasslands and alvars of Northern and Western Estonia deserve special attention as a valuable genetic resource. These populations growing on the northern margin of their distribution range are historically adapted to fluctuating environmental conditions, including cold winters and frequent summer droughts, and have the ability for extensive vegetative spreading in contrast to *sativa* and *varia*.

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SUMMARY IN ESTONIAN

SIRPLUTSERNI *(MEDICAGO SATIVA* SSP. *FALCATA)* LOODUSLIKE POPULATSIOONIDE GENEETILINE MITMEKESISUS, GENOTÜÜPNE STRUKTUUR JA OHUSTATUS EESTIS

Paljud põllukultuurid on võimelised ristuma oma looduslike esivanematega piirkondades, kus nende levilad kattuvad. Tavaliselt on ristumine harv juhus ning sellel ei ole püsivaid tagajärgi. Kuid risttolmlevate (võõrtolmlevate) taksonite hulgas võib põllukultuuride ning nende looduslike eellaste omavaheline tolmlemine ning ristumine olla sage ja hübriidne järglaskond elujõuline. Hübriidide tekkele võib soodustavalt mõjuda ka inimtegevus, kas teadlik looduslikele lähedaste kultuurtaimede sissetoomine või juhulevi inimese kaasabil, mis võib loodusliku ristumisbarjääri lõhkuda. Introgressiivne hübridisatsioon kultuurtaimedelt looduslikesse populatsioonidesse võib looduslikke vorme mitmeti mõjutada. Geenisiirde tagajärjel võivad kujuneda invasiivsete omadustega taksonid, looduslikud vormid võivad geneetilise reostumise tagajärjel kaduda ning populatsioonides hävida kohalikele tingimustele kohastunud alleelid või genotüübid. Samas on leitud, et uued moodustunud genotüübid võivad olla erinevates keskkonnatingimustes vastupidavamad ning muutuvates keskkonnaoludes suurema kohanemisvõimega. Hübriidide esinemissagedus, püsimajäämine ning levikuulatus looduslikes tingimustes sõltub liikide sugulusastmest ning tolmuterade levikust ajas ja ruumis.

Sirplutsern (*Medicago sativa* ssp. *falcata* (L.) Arcangeli) kuulub hariliku lutserni (*M. sativa* ssp. *sativa* L.) ning hübriidlutserniga (*M. sativa* ssp. x *varia* (Martyn) Arcang) samasse *Medicago sativa-falcata* kompleksi, millele on omane sage ristumine looduslike ning kultuurtaimede vahel. Sirplutsernil, harilikul lutsernil ning hübriidlutsernil on ühesugune tetraploidne kromosoomiarv (2n=32), mistõttu nende hübriidid on elujõulised. Harilikul ning sirplutsernil on olemas ka diploidsed vormid (2n=16). Hariliku lutserni diploidne vorm on iseseisev takson taevassinine lutsern (*Medicago sativa* ssp. *caerulea* (Less. ex Ledeb.)). Samal ploidsuse astmel ristuvad need kolm taksonit vabalt ja nende järglased on elujõulised, seega käsitletakse neid *M. sativa* alamliikidena. Taksonite omavaheline edukas ristumine tuleneb ka tolmeldajate, õitsemisaegade

ning levilate kattumisest. Kõikjal, kus nende kolme alamliigi levilad kattuvad ning ploidsusastme tõttu ristumisbarjäär puudub, on ristumine kultuurtaimede ning nende looduslike eellaste vahel väga sagedane.

Eestis on sirplutsern omamaine liik, talle on iseloomulik kollane õievärv ning sirpjad kuni sirged kaunad. Harilik lutsern on Eestisse sisse toodud 19. sajandi keskpaigas. Teda iseloomustavad lillad õied ning spiraalselt keerdunud kaunad. Nüüdseks on ta kultuurist metsistunud ning kasvab hajusalt kogu Eestis. Hariliku ning sirplutserni ristand on kirju õievärvi ning spiraalselt keerdunud kaunadega hübriidlutsern. Eestisse sissetoodud hübriidlutsern on kohalike looduslike tingimustega hästi kohanenud, aja jooksul naturaliseerunud ning levib hajusalt pea kõikjal Eestis. Eestis on kõik kolm alamliiki teadaolevalt autotetraploidsed. Esimesed teated kirjuõielistest hübriidset päritolu taimedest looduslikes sirplutsernipopulatsioonides pärinevad 1970. aastatest.

Kuna looduslik sirplutsern ristub sagedasti kultuurvormidega, tõstatub küsimus geneetiliselt puhaste looduslike lutsernipopulatsioonide püsimisest. Kultuurtaimede looduslikud eellased on väärtuslik materjal uute sortide aretustööks ning nad väärivad looduskaitselist tähelepanu. Suur geneetiline mitmekesisus tagab ka vajalikud eeldused populatsioonide ellujäämiseks ning kohastumiseks muutuvates keskkonnatingimustes. Kasvukohtade hävimist ning fragmenteerumist e. killustumist peetakse olulisteks faktoriteks, mis vähendavad geneetilist mitmekesisust. Killustunud ning isoleerunud populatsioonides hakkavad toimima mitmed negatiivsed mõjud: suurenevad geenitriiv ning lähiristumissurutis, väheneb geneetilise materjali vahetus populatsioonide vahel. Lühema aja jooksul võib väikestes populatsioonides alleelse mitmekesisuse kadu vähendada indiviidide elujõulisust ja kohasust, pikema aja vältel võib vähenenud geneetiline mitmekesisus mõjutada negatiivselt populatsioonide kohanemisvõimet muutuvate keskkonnatingimuste suhtes. Seetõttu on paljud autorid pööranud tähelepanu kasvukohtade killustumise ning geneetilise mitmekesisuse kao omavahelisele seotusele. Paljudel liikidel on leitud, et kasvukohtade killustumisel on geneetilisele mitmekesisusele negatiivne mõju, samas on mitmetel liikidel täheldatud vastupidist, kus geneetilise mitmekesisuse kadu ei ole seotud kasvukohtade pindala vähenemise ning isoleerituse astme tõusuga. Seega on oluline uurida veel säilinud puhaste sirplutsernipopulatsioonide geneetilise varieeruvuse sõltumist

populatsioonide suurusest ning isoleeritusest. Need populatsioonid paiknevad intensiivse inimmõjuga piirkonnas, mida iseloomustab kasvukohtade hävimine ning killustumine inimtegevuse tagajärjel.

Klonaalsete taimede geneetiline ja genotüüpne mitmekesisus on viimastel kümnenditel üha suuremat huvi ja uurimisainet pakkunud. See tuleneb erinevate molekulaarsete markerite kasutusele võtmisest, mis võimaldavad eristada geneteid ning rameteid, selgitada rametite ruumilist paigutust ning genetite suurust rametite arvu ja hõlvatud pindala kaudu, samuti klonaalse ja sugulise paljunemise osakaalu populatsioonides. Teooria kohaselt peaksid mittesugulisel teel levivad taimed olema geneetiliselt vähem varieeruvad kui sugulisel teel paljunevad liigid. Tegelikult on leitud, et vegetatiivselt levivad liigid omavad sama suurt genotüüpset mitmekesisust kui sugulisel teel paljunevad liigid, kuna enamikul klonaalselt levivatest liikidest on erineval määral säilinud ka suguline paljunemine. Geneetilist mitmekesisust aitavad säilitada ka somaatilised mutatsioonid, seemnepank ning geenisiire populatsioonide vahel. Erinevad liikide omadused (eluiga, paljunemisviis) ning kasvukohatingimused loovad heterogeense ruumilise ja geneetilise struktuuri. Sugulise ning vegetatiivse paljunemise osakaal varieerub liigiti oluliselt, sõltudes samal ajal ka keskkonnatingimustest, mille ebasoodne varieeruvus (teatud tingimustel) võib viia sugulise paljunemise kaoni. Seetõttu annavad olulist uut informatsiooni uuringud, mis võrdlevad genotüüpset mitmekesisust ning ruumilist struktuuri ühe liigi populatsioonide sees ning vahel erinevates kasvukohatingimustes.

Uurides Eesti looduslike sirplutsernipopulatsioonide risustumist hariliku ning hübriidlutserni taimedega, selgus, et 106 vaadeldud populatsioonist oli puhtaid vaid 15, s.t. nendes populatsioonides ei kasvanud ei hariliku ega hübriidlutserni taimi. Sirplutserni ristumine kultuuris kasvatatavate hariliku ning hübriidlutserniga looduslikes Eesti sirplutserni populatsioonides on laialdane ning jätkuv protsess, mis toimub nii kimalastest tolmeldajate abil kui seemnete leviku kaudu. Puhastes sirplutsernipopulatsioonides on sirplutsernitaimede morfoloogilised tunnused püsivad, neil on kollased õied ning sirpjad kuni sirged kaunad. Segapopulatsioonides võib üks indiviid omada morfoloogilisi tunnuseid, mis on iseloomulikud nii sirplutsernile, harilikule lutsernile kui ka hübriidlutsernile. Hübriidset päritolu taimede olemasolu populatsioonides ei sõltu küll ei kasvukohast ega

muutustest maakasutuses, kuid kõige soodsamad tingimused hübriidsete taimede levikuks on tugeva inimmõjuga aladel: teeservades, jäätmaadel, söötidel. Loodusliku sirplutserni säilinud puhtad populatsioonid liigi levila põhjapiiril Lääne- ja Põhja-Eesti alvaritel väärivad looduskaitselist tähelepanu kui väärtuslik omamaine, karmidele kasvutingimustele kohastunud geneetiline ressurss.

Veel säilinud puhaste sirplutsernipopulatsioonide suuruse ning isoleerituse astme vahelise seose väljaselgitamisel üheksas populatsioonis ning alampopulatsioonis Paldiski, Kurkse ning Kunda piirkondades selgus, et geneetiline mitmekesisus (H_e) on kõrge kõigis uuritavates populatsioonides, olenemata nende suurusest ning isoleerituse astmest, ulatudes 0.795-0.893. Võrreldes kõige väiksemat populatsiooni (1.5 ha) ligi sada korda suurema populatsiooniga (165 ha) oli geneetilise mitmekesisuse tase peaaegu sama, vastavalt 0.795-0.808. Populatsioonid jagunevad geneetilise distantsi alusel kahte gruppi, mis peegeldab vaid osaliselt nende geograafilist paiknemist. Eraldi grupeerusid Paldiski ning Kurkse piirkonna populatsioonid, kuid eemalasetsev Kunda populatsioon paigutus ühte gruppi Paldiski piirkonna populatsioonidega. Kõrge geneetilise mitmekesisuse uuritavates populatsioonides võib tagada liigi autotetraploidsus, pikaealisus, iseviljastumisel tekkinud homosügootide elimineerimine seemnete moodustumisel ja järglaskonnas ning seemnepangast lisanduvad uued kõrge heterosügootsusega isendid.

Uurides puhaste sirplutsernipopulatsioonide genotüüpset mitmekesisust ning genetite ruumilist paigutust, ilmnes, et genotüüpne mitmekesisus ning genetite ruumiline paigutus varieerusid suures ulatuses nii populatsioonide sees kui vahel ning samuti erinevate uurimisskaalade võrdluses. Analüüsisime isoensüümmarkeritega kuue populatsiooni genotüüpset struktuuri erinevatel ruumilistel skaaladel: 1m^2, 4m^2 ja 30-60 m transektidel. Eeldasime, et uuritavates populatsioonides võiks olla erineva suurusega geneteid, mis on tekkinud seemnelise paljunemise tulemusena lokaalsetes väikseskaalalistes häiringukohtades ning on alustanud seejärel vegetatiivset kasvu. Peamisteks eesmärkideks oli välja selgitada, mil määral varieerub sirplutserni klonaalne mitmekesisus sõltuvalt ruumilisest skaalast ning kuidas on klonaalne struktuur ning mitmekesisus seotud populatsioonide ökoloogilise heterogeensusega. Selgus, et genotüüpne varieeruvus ning genetite ruumiline paigutus varieerub suures ulatuses nii populatsioonide sees kui ka vahel ning

samuti erinevate ruumiliste skaalade võrdluses. Ulatusliku varieeruvuse põhjuseks võib olla vegetatiivse ning seemnelise paljunemise erinev osakaal, mis sõltub kasvukoha ökoloogilistest tingimustest. Leidsime, et üheks selliseks ökoloogiliseks tingimuseks, mis mõjutab seemnelist paljunemist ning sellele järgnevat vegetatiivse kasvu mustrit populatsioonis, võib olla taimkatte tihedus. Taimkatte tiheduse mõju on selgelt näha populatsioonis P6, mida iseloomustab tihe kõrreliste katvus ning paks kevadine kulukiht, mis vähendab võimalusi edukaks seemneliseks paljunemiseks idandite suremise tõttu. Töö tulemused näitavad, et sellised tingimused põhjustavad monoklonaalsust või väga väikest genotüüpset mitmekesisust uuritud ruutudes. Oletame, et loodusliku valiku tagajärjel on populatsioonis lokaalselt kaduma läinud sellesse keskkonda vähemsobivad genotüübid ning alles on jäänud isendid, kes on kohanenud vastavate tingimustega.

Teiseks seemnelist paljunemist soodustavaks tingimuseks on eeldatavalt lokaalsed häiringud. Tõendeid seemnelise paljunemise kohta väikeseskaalaliste häiringute tagajärjel oli võimalik näha populatsioonis P6 uuritud transektil, kus tõenäoliselt väikeste häringute tulemusena on tekkinud sobivad tingimused seemneliseks paljunemiseks. Kontrastsed tulemused olid näha populatsioonis P4 1m^2 prooviruutude võrdluses. Kaks uuritavat ruutu olid peaaegu monoklonaalsed, samas kui ühes ruudus kuulusid peaaegu kõik vaadeldud võsud erinevatesse genotüüpidesse. Ka populatsioonides P7, P8 ja P9 esines kõigis 1m^2 suurustes ruutudes märgatav genotüüpse mitmekesisuse varieeruvus. Seega ka neis populatsioonides on seemnelise paljunemise osakaal oluline, sõltudes lokaalsetest häiringutest ning ka populatsiooni ajaloolisest taustast.

Kokkuvõtvalt võib öelda, et sirplutsern on võimeline paljunema seemneliselt ka suhteliselt tiheda taimkattega looduslikel rohumaadel, kuid selle ulatus, hinnates seda genotüüpse mitmekesisuse kaudu, sõltub lokaalsetest häringutest. Häiringud võimaldavad uute isendite kasvu seemnepangast või iga-aastasest seemnelisest paljunemisest. Erinev populatsioonide ajalugu ning maakasutus ja sellest tulenevad ökoloogilised tingimused seletavad väga suurt varieeruvust genotüüpses mitmekesisuses ning ruumilises struktuuris nii populatsioonide vahel kui ka sees. Võrreldes genotüüpset mitmekesisust erinevatel ruumilistel skaaladel, selgus, et mida väiksem oli skaala, seda suurem oli klonaalsuse määr, millest järeldub, et väga oluline on õige uurimisskaala valik

populatsioonide genotüüpse struktuuri selgitamisel seoses lokaalse ökoloogilise heterogeensusega.

Käesolev doktoritöö tõstatas *Medicago sativa-falcata* kompleksi näitel olulise temaatika kultuurtaimede ning nende looduslike eellaste ristumisest. Selgus, et sirplutserni populatsioonide risustumine kultuuris kasvatatavate hariliku ning hübriidlutserniga looduslikes Eesti sirplutsernipopulatsioonides on laialdane ning jätkuv protsess. Käesolev töö on esimene, mis kirjeldab looduslike sirplutsernipopulatsioonide geneetilist ning genotüüpset varieeruvust sõltuvalt kasvukohatingimustest. Leidsime, et looduslikud sirplutsernipopulatsioonid on kõrge geneetilise varieeruvusega ning geneetilise mitmekesisuse säilitamisel on oluline osa uute genotüüpide lisandumisel seemnepangast ja iga-aastasel seemnelisel uuenemisel.

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I

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Extensive introgressive hybridization between cultivated lucerne and the native sickle medic (*Medicago sativa* ssp. *falcata*) in Estonia

Karin Kaljund* & Malle Leht

*Department of Botany, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, 5 Kreutzwaldi str., EE-51014 Tartu, Estonia (*corresponding author's email: karin.kaljund@emu.ee)*

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We studied the occurrence of the crop-to-wild introgressive hybridization in the *Medicago sativa-falcata* complex in the distribution area of the native *M. sativa* ssp. *falcata* (hereafter *falcata*) in western and northern Estonia. Flower colour and pod shape were used as the diagnostic genetic characters to assess the extent of hybridization from cultivated lucerne to populations of *falcata*. Among the 106 populations examined, only 15 were pure yellow-flowered *falcata* populations. In the remaining 91 populations, 1%–90% of the plants had variegated flowers typical of hybrid plants, indicating widespread but highly variable introgression. Hybrid plants were detected most frequently in disturbed man-made habitats, mostly roadsides, wastelands and fallow fields. Hybrid plants with variegated flowers were found to have not only pod shapes characteristic of *falcata* but also coiled pods characteristic of cultivars, indicating that due to introgression hybrid populations have become morphologically more diverse.

Introduction

Hybridization between domesticated taxa and their wild relatives is quite common under natural conditions in areas of their sympatric distribution and it has been surveyed in many papers (e.g. De Wet & Harlan 1975, Small 1984, Raybould & Gray 1993, Rieseberg & Wendel 1993, Ellstrand *et al*. 1999, Jarvis & Hodgkin 1999, Ellstrand 2003, Hails & Morley 2005, Lane 2005). Introgressive hybridization can negatively affect wild populations in various ways, e.g. by promoting development of aggressive weeds (Ellstrand *et al*. 2010), by genetic modification and assimilation of native wild forms (Riesenberg & Ellstrand 1993, Levin *et al*. 1996, Rhymer & Simberloff 1996, Ellstrand *et al*. 1999, Lane 2005), through outbreeding depression (Hails & Morley 2005) and through possible escape of crop transgenes to natural populations (Lavigne *et al*. 2002, Chapman & Burke 2006, Chèvre *et al*. 2007, Warwick *et al*. 2008).

Likelihood of the gene flow for each crop-weed or crop-wild relative combination is different and depends on many factors (Lane 2005). Therefore, for successful hybrid formation some important assumptions must be fulfilled. The crop and its wild relative should grow in close proximity, they should share the same pollinators, their flowering times should overlap and

their hybrids should be viable and fertile (Chapman & Burke 2006). As the gene flow from cultivated plants to natural ones is mainly pollen-mediated, it can range over long distances (Raybould & Gray 1993, St. Amand *et al*. 2000, Lane 2005). In addition, cultivated alfalfa has been shown to disperse by seeds and to be able to establish itself in roadside habitats (Bagavathiannan *et al*. 2010) were it is able to hybridize with the native sickle medic (e.g. Jenczewski *et al*. 1999a, 1999b).

The *Medicago sativa-falcata* complex is an excellent example for examining hybridization between a crop and its wild form. *Medicago sativa* ssp. *falcata* (= *M. falcata*, sickle medic, hereafter *falcata*) is characterised by yellow flowers and sickle-shaped pods, while *M. sativa* ssp. *sativa* (hereafter *sativa*) has purple flowers and highly coiled pods (Lesins & Lesins 1979). Their hybrid is called *M. sativa* ssp. *varia* (hereafter *varia*), and it also has coiled pods but its flowers are variegated. The flower colour can be mixed purple-yellow, whitish, and greenish, even brown (Small & Brookes 1984). At the same ploidy level (2n = 32), the three taxa mate freely producing fertile hybrids and are therefore treated as subspecies of *M. sativa s. lato* (Small & Brookes 1984, Quiros & Bauchan 1988). Although *falcata* and *sativa* have also diploid forms (2n = 16), diploid *sativa* is considered a separate subspecies, *M. sativa* ssp. *caerulea*, while the diploid forms of *falcata* have not been recognized as a separate taxon (Small 1984). Whereas crossing is more difficult between ploidy levels, formation of unreduced gametes in diploids is frequent (Barcaccia *et al*. 2003, Small 2011) thus making hybridization possible between diploid and tetraploid taxa.

Extensive hybridization between the native and cultivated forms of *sativa* has been observed in Spain (Jenczewski *et al*. 1999a, 1999b, Muller *et al*. 2001, Prosperi *et al*. 2006) and Kazakhstan (Greene *et al*. 2008), whereas in Switzerland (Rufener Al Mazyad & Ammann 1999) and Germany (Bleeker *et al*. 2007) frequent hybridization between native *falcata* and cultivated *sativa* and *varia* has been recorded. It was concluded that natural populations of *falcata* in Europe are under strong introgression pressure from cultivated *sativa* and *varia* (Muller *et al*. 2003).

Medicago sativa ssp. *falcata* is native to Estonia, being mostly distributed on calcareous grasslands, alvars (calcareous grasslands on limestone plain with thin soil), and also along roadsides and on wastelands (Kukk 1999) in western and northern Estonia. *Medicago sativa* ssp. *sativa* was introduced to Estonia in 1830 (Miljan 1932). At present, *sativa* has escaped from cultivation and has become partly naturalized, growing all over the country, but more often in western Estonia (Kukk & Kull 2005). Cultivated *varia* is widely distributed throughout Estonia, being well adapted to the local natural conditions (Kukk 1999, Kukk & Kull 2005). Different introduced cultivars of *sativa* are not as resistant to the Estonian climate as are cultivars of *varia*, which have become more readily naturalized. During the 150-year cultivation period, ample diverse plant material has been introduced to Estonia from western Europe and North America, and even from the surroundings of the Caspian Sea and the Black Sea (Bender & Tamm 1998). Thus, highly different genetic material of alfalfa cultivars, able to cross with the local native sickle medic, has been accumulated in Estonia. The first notes about such hybrid swarms were published in the 1970s and 1990s (Bender & Tamm 1998).

The general goal of this study was to assess the occurrence of crop-to-wild gene flow in the *M. sativa-falcata* complex in Estonia. Our specific objectives were: (1) to describe the distribution of hybrid plants among Estonian natural *falcata* populations, (2) to find out how hybridization has influenced two morphological characteristics (flower colour, pod shape) in *falcata* mixed populations, (3) to clarify if habitat type has any influence on the occurrence of hybrid plants, (4) to elucidate if changes in land use have affected the abundance of hybrid swarms.

Material and methods

Studied taxa

The subspecies *falcata*, *sativa* and *varia* are all perennial, autotetraploid (2n = 32), cross-pollinated (Lesins & Lesins 1979) and partially self-incompatible (Viands *et al*. 1988). Although

Fig. 1. Distribution map of the studied 106 *Medicago sativa* ssp. *falcata* populations in Estonia. Black triangles denote mixed populations and black circles denote pure populations of *falcata*.

falcata and *sativa* have also diploid forms (2n = 16), in the Estonian native populations so far only tetraploid *falcata* has been found (Kaljund & Leht 2010). Their primary pollinators are bumblebees (*Bombus* spp.) and solitary bees (*Megachile* spp., *Andrena* spp. and others) (Martin *et al*. 1998). The growing conditions for the three taxa are quite similar: they prefer calcareous light soils on road sides, meadows, and wastelands (Lesins & Lesins 1979). In Estonia, *falcata* is also growing in such natural habitats as dry calcareous grasslands. The native sickle medic is more tolerant of winter and grazing than *sativa* or *varia* (Lesins & Lesins 1979), while its forage yield is poorer (Riday & Brummer 2004). In Estonia, cultivated *varia* is well adapted to the natural conditions and is therefore also widely naturalized, while *sativa* persists in natural conditions near cultivated fields only for a short time.

Study sites

The study was carried out in 2008 and 2009 in the distribution area of the native sickle medic in western and northern Estonia. A total of 106 populations were sampled (Fig. 1). The populations studied were growing mostly in open habitats under dry or semi-dry conditions. Of the 106 populations studied 37% grew on roadsides, 27% were settled on grasslands, 16% on alvars, 13% on fallow fields, and 7% were growing on wastelands.

To describe how hybridization has influenced the morphology of the plants in the studied populations, two diagnostic morphological characteristics were recorded: flower colour and pod shape. As noticed earlier, these characters are the most informative for detecting hybrids in the *M. sativa-falcata* complex (Small & Brookes 1984). Flower colour and pod shape are genetically-determined characters (Barnes & Hanson 1967). The purple flower colour pigments of *sativa* are determined by one gene with tetrasomic inheritance. The yellow flower colour of tetraploid *falcata* is caused by at least two genes with accumulative effects (Barnes 1966). Yellow flower colour, sickle-shaped pods, straight pods and corkscrew-twisted pods were assigned to *falcata* and variegated flowers (pale-yellow, greenish-yellow, mixed purple-yellow and brownish) and coiled pods were attributed to plants of hybrid origin.

To estimate how habitat type and changes in the land use may have favoured the distribution of hybrid plants, in each population the mean coverage percentage of *Medicago* plants was estimated separately for the yellow-flowered sickle medic and for plants with variegated flowers. The changes in the land use, caused by human activity, were recorded based on aerial photographs from the 1950s, which were com-

pared with the current situation as described for 67 populations during our fieldwork.

Data analyses

The data were analysed with the statistical software STATISTICA (StatSoft Inc.). One-way ANOVA followed by Tukey's post hoc test was used to test whether the differences between the population characteristics (habitat type and land-use changes in the studied populations) and percentage of hybrid plants in populations are significant. The relationship between flower colour and pod shape was tested using the χ^2-test.

Landscape analysis was applied to estimate changes in habitat types. Land use changes in the studied populations were described by interpretation of aerial photos (large-scale ortophoto maps 1:10 000) from 1951–1957 using the mapping package MapInfo Professional (MapInfo Corporation 2004).

Results

Among the 106 populations studied only 15 were pure *falcata* populations without *varia* or *sativa* plants (Fig. 1). In the remaining 91 populations, 1%–90% of the plants had variegated flowers, indicating widespread but highly variable introgression. Pure populations occurred on alvars (six), on dry grasslands (four), along roadsides (three), on a fallow field (one) and one inhabited an old sandpit.

In the studied populations, the flower colour and the pod shape varied on a large scale, and the flower colour varied independently of the pod shape. According to the χ^2-test, yellow-flowered *falcata* plants never had coiled pods. However, plants with variegated or pale yellow flowers may have pods characteristic of *falcata*, or coiled pods ($\chi^2_1 = 9.1$, $p = 0.002$). Some plants had intermediate morphological characters as compared with those of their parental taxa. Those plants had greenish-yellow flowers and pod shapes characteristic of *falcata* or *varia*. The greenish-yellow flower colour indicates first-generation hybrids between *falcata* and *varia* (Small & Brookes 1984). Comparison of hybrids with greenish-yellow flowers and yellow-flowered *falcata* revealed that plants with yellow flowers had never coiled pods, whereas hybrids had coiled pods or pods characteristic of *falcata* equally frequently ($\chi^2_1 = 9.3$, $p = 0.002$). Comparison of hybrids with greenish-yellow flowers with hybrids that had variegated flowers revealed that flower colour and pod shape were not associated. Hybrid plants with variegated or greenish-yellow flowers had coiled pods or pods characteristic of *falcata* equally frequently ($\chi^2_1 = 0.68$, $p = 0.4$). Occurrence of hybrid plants was not dependent on the habitat type ($F_{4,101} = 0.8$, $p = 0.53$). However, on roadsides and wastelands the percentage of hybrid plants in a population was on the average 20–25, which is higher than that in the other habitat types.

Land-use changes had no influence on the abundance of hybrid plants ($F_{1,65} = 1.98, p = 0.16$). In populations where land use has changed, the average percentage of hybrid plants was higher, but that difference was not statistically significant. In populations where land use has remained the same, the average percentage of hybrid plants was 14%; in populations with changed land use it was on average 22%. Land use changes took place in 64% of the studied populations. According to the maps from 1951–1957, the investigated populations inhabited the following habitats: pastures, grasslands, forest, fields, shrubs, and fallow fields. The observations made during the fieldwork showed that only 9% of these pastures are nowadays still used for grazing, 38% of the former pastures are now grasslands which are still mowed, and 48% are abandoned. Of the former pastures 5% are wastelands (Fig. 2). Two former grasslands are used nowadays as grasslands. One former grassland and one former field are now abandoned sandpits. Of the former fields, 57% are nowadays fallow fields and 28% are managed grasslands. Wastelands, overgrown fields and unmanaged grasslands account for 5% each (Fig. 2). The area of two populations was in the years 1951–1957 overgrown with shrubs. Today one of these populations is an abandoned grassland and the other is a wasteland. One former fallow field is used today as a grassland. In 36% of the populations, land use is the same as it was 60 years ago. They are mostly roadsides (84%), grasslands (8%) and pastures (8%).

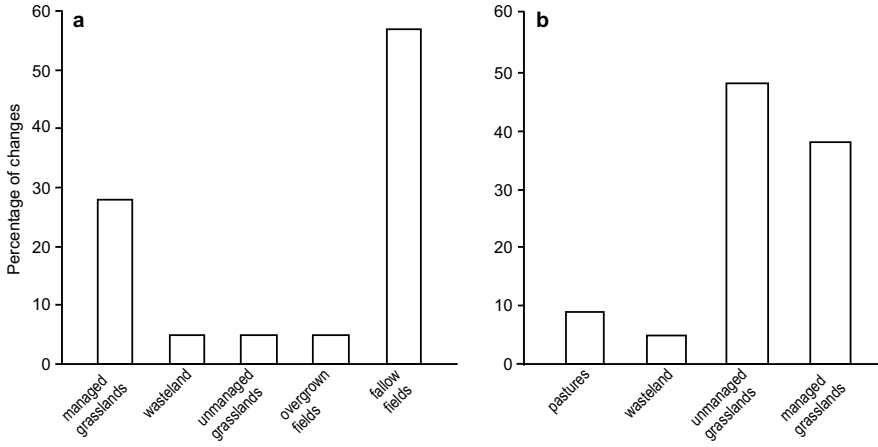

Fig. 2. Land-use changes in (**a**) fields and (**b**) pastures during the past 60 years.

Discussion

Variation in diagnostic morphological traits

Hybrid plants in the *M. sativa-falcata* complex have traditionally been identified on the basis of flower colour and pod shape (Small & Brookes 1984, Jenczewski *et al.* 1998). We also used flower colour and fruit characteristics to identify hybrid plants. To distinguish hybrid plants, one should examine both flower colour and pod shape, because of their independent variation among individuals. A confusing and limiting circumstance in the interpretation of the data obtained was that hybrid plants are sometimes hard to distinguish on the basis of morphological characters. Moreover, two Estonian *varia* cultivars bred for pastures have pale-yellow flowers and their pods may vary from sickle-shaped to spirally-coiled (Bender 2006). In addition, the pod characteristics of *falcata* are variable: pods can be occasionally twisted like a corkscrew (Lesins & Lesins 1979, Quiros & Bauchan 1988). In Estonia, owing to morphological similarities between natural *falcata* and pale yellow-flowered *varia*, the latter has sometimes been misidentified as *falcata*. According to the latest distribution map (Kukk & Kull 2005), the distribution area of *falcata* has expanded to southern and southeastern Estonia, but these more southeastern localities are evidently inhabited by yellow-flowered *varia*.

Earlier studies have indicated a continuum of variation among *falcata*, *sativa* and *varia*, but intermediacy between *falcata* and *varia* is smaller. More complete intergradation occurs between *sativa* and *falcata* (Small & Brookes 1984, Quiros & Bauchan 1988). We also found that flower colour and pod shape varied in the observed populations on a large scale, however, there was no strong association between them. We found that yellow-flowered *falcata* plants never had highly-coiled pods but they were quite often corkscrew-twisted. Plants with variegated flowers may have pods characteristic of *falcata*, or even coiled pods characteristic of *sativa*. It has been reported (Small & Brookes 1984) that stabilized recombinant populations with odd character associations, for example, yellow-flowered plants with coiled fruits are frequent in some parts of the world. Obviously, the Estonian plants with pale yellow flowers and coiled pods belong to *varia* and plants with variegated flowers are hybrids with introgressed traits of *falcata*.

Yet no specific molecular markers were found to distinguish between cultivated and wild pools, but the populations of wild and cultivated plants were genetically distinguished by allele frequencies (Jenczewski 1998, Jenczewski *et al.* 1999a,

1999b, Muller *et al*. 2001, Greene *et al*. 2008). On the other hand, diploid forms of ssp. *falcata* and ssp. *sativa* were well distinguishable using SSR markers (Şakiroğlu *et al*. 2010) and cpDNA sequences (Havananda *et al*. 2010). Because of the large overlapping of morphological characteristics and molecular data in the *M. sativa-falcata* complex, both selected molecular markers and morphological characters are needed to study introgressive hybridization in more detail.

Distribution of hybrids in populations depending on the habitat type and changes in land use

Human activities often give rise to ruderal land which can easily be colonized by numerous taxa, mostly species with a high colonization ability. Habitat disturbance creates suitable habitats for hybrid taxa and increases opportunities for spontaneous hybridization. In disturbed habitats, formerly separated taxa may come into contact as human activities have destroyed ecological and spatial barriers (De Wet & Harlan 1975, Levin *et al*. 1996). It is also known that hybrid taxa are most successful in disturbed habitats but usually not in habitats preferred by their parental taxa (De Wet & Harlan 1975).

In breeding programs, *falcata* has been intensively used for improving winter hardiness and grazing tolerance of *varia* cultivars. In Estonia, two grazing-tolerant yellow-flowered *varia* cultivars have been bred. As a result, differences in the requirements for habitat and environmental conditions between *falcata* and *varia* have diminished, and domesticated plants of *varia* are able to survive and spread under natural conditions. This is well evident in Canada were roadside feral populations of escaped cultivars with high parentage from *falcata* are very viable (Bagavathiannan & Van Acker 2009, Bagavathiannan *et al*. 2010). These populations seem to be under natural selection for winter survival and clonal growth. It was assumed that these traits of *falcata* may support persistence of roadside hybrid populations (Bagavathiannan & Van Acker 2009, Bagavathiannan *et al*. 2010). Even more first-generation hybrids between *sativa* and *falcata* show the effects of heterosis i.e., vigorous growth, larger plants and a more erect habit (Riday & Brummer 2004). In Estonia, the traits of *falcata* may support the establishment of hybrid plants in natural populations on alvars and dry grasslands in addition to roadside habitats, as only six populations growing on alvars appeared to be pure.

Besides alvars and dry grasslands, three pure *falcata* populations were growing at roadsides, one pure population occurred on a fallow field and one pure population in an old sandpit. Presumably, these populations were recently established by the initial seedling recruitment from *falcata* seeds. It may be expected that in the course of time, hybrid plants will appear also in these populations from the nearby cultivated fields or as a consequence of human activity (e.g. road-construction works). It has been shown that gene flow by pollen from cultivated *sativa* and *varia* fields may cover long distances (St. Amand *et al*. 2000). Furthermore, also gene flow via seeds can be essential and may increase gene exchange between natural and cultivated populations (Muller *et al*. 2001). We suppose that there may be three main reasons why hybrid plants are missing from some populations: (1) unsuitable environments for dispersal of hybrid plants, (2) lack of cultivated fields nearby natural populations of *falcata*, (3) populations in disturbed habitats that have been established by *falcata* seeds are now densely occupied by plants of *falcata* and other competitive species. As the results of this study show that habitat type had no influence on frequency of hybrid plants, the impact of other factors, e.g. those mentioned above, which do not support spreading of hybrid plants, may play an important role.

Prevalent processes in the Estonian agricultural landscapes are forestation, abandonment and overgrowing of grasslands with shrubs and trees, and expansion of fallow fields in formerly arable land (Kana *et al*. 2008). This is in accordance with our observations which also confirm that former grasslands, pastures and arable fields are often abandoned and overgrown by shrubs. In these unmanaged areas the dispersal of competitive and large-sized invasive species, for example *Galega orientalis* and *Lupinus polyphyllus*, is promoted (Eek & Kukk 2008).

In many cases, it is not known if *falcata* and *varia* are growing on pastures and grasslands nat-

urally or have been sown there during the period of intensified agricultural practices since 1950 until 1990. At many sites, plants sown in that period have persisted until now (Bender 2006). One can claim that cultivation of grasslands and pastures has historically contributed to better distribution of hybrid plants, making co-occurrence of the two taxa possible. Also abandonment of sandpits and former arable fields has enabled both *falcata* and *varia* to colonize new open areas. However, we found no significant difference in the occurrence of hybrid plants at sites with changed land-use as compared with sites where the land use had remained the same, although escaped cultivars obviously have more favourable conditions for spreading at roadsides. It is evident that *varia* has adopted traits of *falcata* which support persistence of hybrid plants in man-made as well as in natural habitats, largely owing to the abandonment of former agricultural areas.

Conclusions

In summary, the present study on hybridization in the *Medicgo sativa-falcata* complex showed that there have remained only a few pure *falcata* populations in Estonia; out of the 106 populations studied by us only 15 were pure *falcata* populations, while the remaining 91 populations contained hybrid plants. These results indicate ongoing and variable hybridization in natural populations of *falcata*. The still preserved pure populations are of high conservation value because introgression from cultivated alfalfa is reported throughout Europe (e.g. Jenczewski *et al*. 1999a, Rufener Al Mazyad & Ammann 1999, Bleeker *et al*. 2007).

The occurrence of hybrid plants in the populations does not depend significantly on the habitat type and is not strongly influenced by changes in land use. However, it can be argued that the most favourable habitats for the growth and dispersal of hybrid lucerne plants are disturbed man-made habitats, mostly roadsides, but also wastelands and fallow or abandoned fields. Pure populations of *falcata* contain only individuals with yellow flowers and sickle-shaped pods, while hybrid plants in mixed populations have individuals with a pod shape and flower colour characteristic of *falcata*, *sativa* or *varia*. The habitat preferences and morphological characteristics of *varia* and *falcata* have become more diverse in hybrid plants through introgressive hybridization in mixed populations, favouring their invasion to disturbed and natural habitats. In addition, pure *falcata* populations on calcareous grasslands and alvars of northern and western Estonia, growing on the northern margin of the distribution range, should deserve special attention as a valuable genetic resource adapted to cold winters and frequent summer droughts, and because they can spread vegetatively. *Ex situ* conservation of pure *falcata* gene pool is carried out in Estonia by the Jõgeva Plant Breeding. In 2002–2009, expeditions were carried out in north and west Estonia to collect seeds from natural populations of *falcata* and other legumes and grasses. Our previous study on the genetic diversity in small and fragmented pure populations of the sickle medic in Estonia showed that it still has remained high and deserves protection (Kaljund & Jaaska 2010). Therefore, *in situ* conservation measures of these populations should be considered, such as a ban of cultivation of *sativa* and *varia* in the radius of possible gene flow distance, and preserving remained populations from destruction through human activities. But even then, the populations are not fully protected against gene flow from cultivated crops due to pollinator-mediated gene flow or occasional long-distance seed dispersal of cultivars. Thus, the conservation measures of *falcata* in Estonia are still moderate and in the future *falcata* deserves more attention as a valuable genetic resource.

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No loss of genetic diversity in small and isolated populations of *Medicago sativa* subsp. *falcata*

Karin Kaljund*, Vello Jaaska

Department of Botany, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, 181 Riia Street, 51014 Tartu, Estonia

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ABSTRACT

Molecular allozyme markers of three polymorphic isozymes were used to estimate the genetic diversity among the seed progeny in fragmented Estonian populations of sickle medic *Medicago sativa* ssp. *falcata* L. depending on the population size and the isolation degree. Genetic diversity H_e was high in all populations, ranging between 0.795 and 0.893. No correlation between the genetic diversity measures and population size or isolation distance was found. Even the smallest population had equally high genetic diversity as about a hundred times larger population. Genetic differentiation of populations into two major groups was associated with the geographic position of populations, except one remote population. Elimination of seed progeny of reduced fitness by embryo abortion and continuous yearlong contribution of the highly heterozygous progeny through the soil seed bank are considered as important supplementary factors that have contributed to maintaining high levels of genetic diversity in populations of sickle medic in addition to its autotetraploid nature and perennial life form.

1. Introduction

Nowadays, habitat destruction and fragmentation are considered as a main factor for reducing species richness and genetic diversity of populations (Saunders et al., 1991; Young and Clarke, 2000; Lienert, 2004). During fragmentation a large continuous habitat divides into smaller patches, causing following effects: reduction in the habitat amount, increase in the number of habitat patches, decrease in sizes of habitat patches, and increased isolation of patches (Fahrig, 2003; Hobbs and Yates, 2003). Population size is considered as an important factor for the maintenance of genetic variation. Genetic diversity is one level of the biological diversity which has a great importance for conservation planning. Therefore, a lot of studies have paid attention on relationships between the population size, degree of isolation and genetic diversity, and the results are discussed in many review papers (Young et al., 1996; Frankham, 1996; Montgomery et al., 2000; Honnay and Jacquemyn, 2007; Leimu et al., 2006, Aguilar et al., 2008; and references therein).

Small and isolated populations formed through the habitat fragmentation are subjected to increased extinction risk through demographic, environmental and genetic stochasticies, being affected by several negative effects, which reduce individual fitness and population viability (Ellstrand and Elam, 1993; Lynch et al., 1995; Matthies et al., 2004). One negative effect of habitat fragmentation is increased genetic drift in small populations, resulting in random losses of rare alleles and reduced genetic diversity. Moreover, higher levels of inbreeding are expected in small populations, caused by increased selfing and mating among closely related individuals, leading to increased homozygosity and decreased individual fecundity due to inbreeding depression (Ellstrand and Elam, 1993; Buza et al., 2000; Dudash and Fenster, 2000; Keller and Waller, 2002).

* Corresponding author. Tel.: +372 7311 874; fax: +372 7383 013.
E-mail addresses: karin.kaljund@emu.ee (K. Kaljund), vello.jaaska@emu.ee (V. Jaaska).

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In short term, a loss of allelic richness can reduce individual fitness and viability of small populations. In longer term, reduced genetic diversity can limit ability to respond to environmental changes through adaptive evolution in the future (Young et al., 1996).

However, the assumptions about the negative effects of fragmentation into smaller and isolated populations seem not be always valid, and the loss of genetic diversity due to fragmentation appears diverse among plant species. Many studies have indeed found positive correlation between the fragmentation and reduced genetic diversity as predicted by the population genetic theory, especially for short-living annuals (Honnay and Jacquemyn, 2007; Leimu et al., 2006). However, several other works have not observed a loss of genetic diversity in small and isolated populations caused by habitat fragmentation for herbaceous perennials (e.g. Young et al., 1999, Pluess and Stöcklin, 2004; Leimu and Mutikainen, 2005; Kuss et al., 2008), shrubs and trees (e.g. Young et al., 1993; Prober and Brown, 1994). These studies show that genetic diversity in fragmented populations may be influenced by certain other factors besides the population size and isolation, indicating a need to investigate more species of different life forms and growing in different habitats before sound generalizations can be made.

In this paper we investigate the effect of habitat fragmentation on the genetic diversity in relation to reduced population sizes and increased isolation in the out-crossing perennial herbaceous legume *Medicago sativa* ssp. *falcata* (L.) Arcang. s. str. (=*M. falcata* L., sickle medic, hereafter ssp. *falcata*), growing on calcareous light-soil grasslands (alvars) and meadows in North and West Estonia. These populations are under strong introgression pressure from cultivated alfalfa *M. sativa* ssp. *sativa* L. (hereafter ssp. *sativa*) and cultivated hybrid lucerne *M. sativa* ssp. × *varia* (Martyn) Arcang., hereafter ssp. *varia*, as also reported earlier for Spain (Jenczewski et al., 1999a; Prosperi et al., 2006), Turkey (Small and Bauchan, 1984) and Switzerland (Rufener Al Mazyad and Ammann, 1999). Evidently, introgression threatens extinction of native ssp. *falcata* in all these regions throughout Europe. Cultivated alfalfa was brought to Estonia at the end of 18th and at the beginning of 19th century and has become increasingly naturalized and hybridized with the native ssp. *falcata*. Our recent fieldworks have shown that yellow-flowered plants of ssp. *falcata* grow mostly in mixed populations together with plants which have differently colored (variegated) flowers typical of hybrid lucerne and purple flowers typical of *sativa* throughout Estonia (Kaljund and Leht, unpublished observations), indicating extensive, ongoing hybridization between native ssp. *falcata* and introduced ssp. *sativa*. We found only a limited number of pure populations with only yellow-flowered plants of ssp. *falcata*, or with very few hybrids of ssp. × *varia*. The native ssp. *falcata* has thus become a rare and threatened taxon in Estonia that needs protection against full extinction.

The habitat area of ssp. *falcata* in Estonia has decreased during the last century by fragmentation and destruction of dry calcareous grasslands (alvars) and meadows where the sickle medic grows. While the area of alvar grasslands in Estonia was estimated more than 43 000 ha in the 1930-ies (Laasimer, 1965), the inventory made between 1979 and 1981 recorded only about 16 000 ha, amounting in a 2.7 times decrease during the last 50 years (Aug and Kokk, 1983). In addition, the remaining calcareous grasslands have become fragmented into habitat patches of various sizes and isolation distances, mainly due to successional changes by natural forestation and increasing human activities.

Genetic variation and differentiation within and among wild and cultivar populations of the *M. sativa* complex has been studied with the use of allozymes (Quiros, 1982; Birouk and Datter, 1989; Jenczewski et al., 1998, 1999a,b; Morales Corts and Crespo Martinez, 2000) and various DNA fragment markers, such as RFLP (Brummer et al., 1991), RAPD (Ghérardi et al., 1998; Jenczewski et al., 1998, 1999b; Mengoni et al., 2000), SSR (Mengoni et al., 2000; Flajoulot et al., 2005; Greene et al., 2008), ISSR (Touil et al., 2008) and AFLP (Greene et al., 2008). Subspecies *falcata* and *sativa* were found to share same sets of allozymes and RAPD bands, but their populations could be characterized by different isozyme allele and some rare RAPD band frequencies (Quiros, 1982; Ghérardi et al., 1998; Jenczewski et al., 1998). However, none of the studies have addressed the effect of anthropogenic fragmentation on the genetic diversity in relation to the size and isolation of remnant sickle medic populations. The general aim of our study is to examine relationships between population size, isolation, genetic diversity and differentiation among the Estonian remnant populations of *M. sativa* ssp. *falcata*. Specific questions are: (1) Is there a relationship between the population size and allozyme genetic diversity? (2) Is there a relationship between the population isolation distance and allozyme genetic diversity? (3) Is the population fragmentation and geographic position related with the genetic differentiation between populations?

2. Materials and methods

2.1. Study species

M. sativa ssp. *falcata*, sickle medic, is a perennial medic that belongs to the *M. sativa* s. l. species complex together with *M. sativa* ssp. *sativa* (=*M. sativa* s. str., alfalfa or lucerne). The taxonomy of the group remains controversial because of different circumscription of taxa in different taxonomical treatments, mostly either at the level subspecies (e.g., Ball, 1968; Small and Brookes, 1984) or distinct species (e. g., Lubenets, 1972; Lesins and Lesins, 1979; Tzvelev, 1987). Alfalfa is clearly distinguishable from ssp. *falcata* by the flower color (blue-purple versus yellow) and pod shape (a spiral of 2–3 turns versus falcate). However, in adjacent and sympatric populations they hybridize readily forming a morphological continuum of fully fertile, segregating hybrids which have been taxonomically treated either as *M.* × *varia* or *M. sativa* ssp. × *varia* (Martyn.) Arcang. (hybrid lucerne). Cytological and genetic studies have shown that ssp. *falcata* and *sativa* s. str. are both autotetraploids with $2n = 32$ (Stanford, 1951; Quiros, 1982).

M. sativa s. l. is an insect-pollinated plant, with bumblebees (*Bombus* spp.) and solitary bees (*Megachile* spp., *Andrena* spp. and others) as the primary pollinators (Martin et al., 1998). *Bombus lucorum* was found to be the most abundant pollinator, making up to 95% of all pollen collectors on flowers of ssp. *falcata*, *varia* and *sativa* in fields located in different regions of Estonia (Martin et al., 1998). Of a total 15 species recorded, *Bombus pascuorum*, *Bombuslapidarius* and *B. lucorum* were the three most abundant bumblebee species in semi-natural and agricultural habitats in Estonia (Mänd et al., 2002), but the relative abundance of the three species varies between years and localities. Bees visiting the flower to collect nectar or pollen open the stigma for pollination using a "tripping" mechanism (Brink and Cooper, 1936). Without insect pollination, the flowers will be self-pollinated, which will lead to increased seed abortion and inbreeding depression of the selfed progeny, presumably through the expression of lethal or deleterious alleles in homozygous condition (Cooper et al., 1937; Whitehead and Davis, 1954; Busbice, 1968).

2.2. Study sites and populations

The study was carried out on natural populations of ssp. *falcata* situated in North Estonia along the coast of the Finnish gulf, growing on natural and semi-natural grasslands (alvars) with calcareous light soils (Fig. 1). The main study area is situated in North-East Estonia around town Paldiski and extends 20 km westward to village Kurkse. The whole region is influenced through strong human impact, with main effects of fragmentation and habitat destruction. The Paldiski region was a closed Soviet military zone from 1940 until 1995. Because of this long period of isolation, pure populations of ssp. *falcata* have maintained in the region without introgression from cultivated ssp. *sativa* or ssp. *varia*. The ssp. *falcata* populations grow in small, remnant grassland fragments that are separated from each other by degraded habitats, buildings and roads.

Populations are delimited as reproductively isolated from each other through gene flow by bumblebee pollination at distances exceeding maximum foraging ranges of *Bombus* spp. which are reported to vary from 450 m for *B. lapidarius* and *B. pascuorum* to about 760 m for *Bombus terrestris* (Knight et al., 2005). The seven populations studied are designated P1–P7 and are characterized by the size and isolation distances in Table 1. Size of the populations ranged from 1.5 ha to 165 ha. Population size was estimated in hectares since it was impossible to count the exact number of individuals because shoots of neighbouring ssp. *falcata* plants are growing interweaving and genets can't be distinguished from ramets. However, the populations studied displayed similar densities of sickle medic plants, and thus the fragment area sizes should be satisfactory measures of population sizes. Distances between populations ranged from 1 to 20 km. The isolation distances between

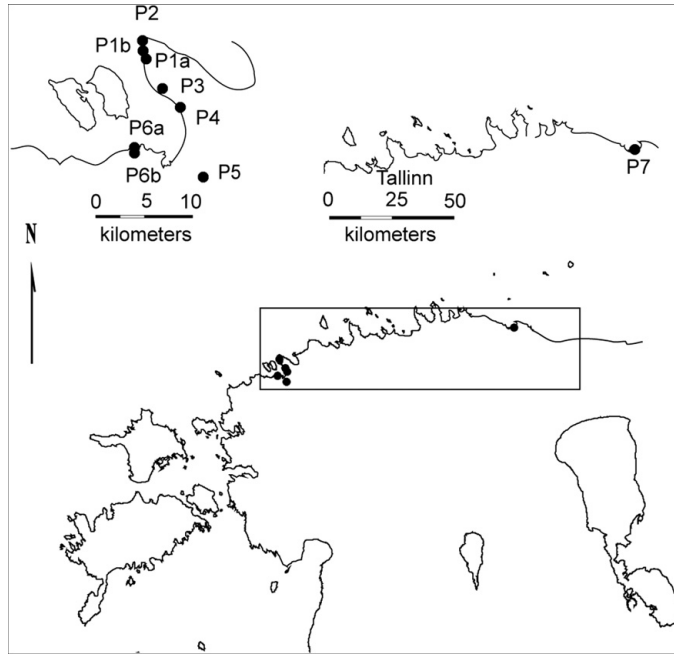

Fig. 1. Map of the nine sampling sites of *Medicago sativa* ssp. *falcata* in North Estonia.

Table 1
Population characteristics and mean population genetic diversity measures for three polymorphic loci in seven populations of *Medicago sativa* ssp. falcata. Standard deviations are given in the parentheses.

Population	*S* (ha)	*I* (km)	*N*	*A*	A_e	H_o	H_e	*F*
P1a	20	0.8	86	3.7	2.1	0.774 (0.14)	0.828 (0.11)	0.065 (0.11)
P1b	15	0.8	54	3.7	2.1	0.827(0.08)	0.840 (0.10)	0.015 (0.03)
P2	15	1.5	63	4	2	0.819 (0.02)	0.840 (0.02)	0.025 (0.01)
P3	1.5	3	69	4.7	2	0.794 (0.08)	0.795 (0.14)	0.002 (0.10)
P4	165	3	52	4	2	0.804 (0.12)	0.808 (0.12)	0.006 (0.01)
P5	4	1	62	3.3	2.3	0.803 (0.12)	0.893 (0.06)	0.103 (0.07)
P6a	1	0.5	68	3.3	2.1	0.838 (0.06)	0.838 (0.10)	0.001 (0.08)
P6b	1	0.5	73	3.7	2.1	0.763 (0.19)	0.843 (0.11)	0.095 (0.11)
P7	5	4	89	3.7	2.1	0.722 (0.19)	0.831 (0.19)	0.136 (0.16)

S – population size (area, in ha)
I – isolation by the distance to the nearest population, km
N – number of individuals analysed
A – mean number of alleles for three loci
A_e – effective number of alleles
H_o – observed heterozygosity
H_e – expected heterozygosity
F – Wright's inbreeding coefficient

populations and subpopulations ranged from 0.5 to 3 km. Population isolation is measured as an edge-to-edge distance to the nearest population.

The populations are situated in three districts: P1a, P1b – P2 – north from town Paldiski, P3–P6 – westward from town Paldiski to the village Kurkse and P7 – north-west of town Kunda, about 110 km eastward from the Estonian capital Tallinn. Populations P1a, P1b, P2 and P3 are isolated from each other by buildings of Paldiski. P1 begins from the northern suburbs of Paldiski and consists of two fragments, P1a and P1b on both sides of a road, separated from each other at about 0.8 km and are considered as subpopulations. Similarly, P6 consists of two subpopulations, P6a and P6b, separated from each other at about 0.5 km. In the Kurkse (P6) and Kunda (P7) populations some minor introgression was noticed with only few plants of variegated flower color.

The number of populations studied was limited to seven because our field studies failed to find any more populations of pure, native ssp. *falcata*: all other populations throughout study area in North Estonia showed many plants of ssp. *varia* with variegated flowers, indicating extensive introgression from nearby fields of cultivated ssp. *sativa* and insufficient genetic isolation (Kaljund and Leht, unpublished data).

2.3. Electrophoretic allozyme analyses

Seeds for allozyme analyses were collected as mature pods from a single branch of individual mother plants separated from each other at least 3–5 m. From each population and subpopulation 52–89 seedlings grown from seeds of 12–15 mother plants were analysed. The pods were sampled throughout populations and subpopulations. Filled seeds collected in September were maintained in a refrigerator at 2–5 °C until next March, then scarified with sandpaper and germinated for two days on two layers of watered filter paper in Petri dishes at 27 °C in a thermostat and thereafter in a growth chamber (12 h light at 25 °C:12 h dark at 15 °C) for additional 3–4 days. Enzyme extracts for allozyme analyses were made by crushing the seedlings at the cotyledon stage individually in 0.35 ml aliquots of the extraction buffer consisting of 0.05 M Tris and 0.01 M EDTA, with 0.01 M thioglycerin added as a sulphydryl protector. After adding about 50 mg of a sucrose – Sephadex G-200 mixture to each extract to increase viscosity and provide cryoprotection, the extracts were stored frozen at −18 °C until electrophoresis in vertical polyacrylamide gel slabs.

The following four enzymes, isocitrate dehydrogenase (IDH, EC 1.1.1.42), phosphoglucoisomerase (PGI, EC 5.3.1.9), peroxidase (PRX, EC 1.11.1.7) and shikimate dehydrogenase (SKD, EC 1.1.1.25), were assayed for allozyme diversity using the following gel–buffer system: 10% acrylamide, 0.2% *N*,*N*′-bisacrylamide (Bis), 0.25 M Tris, and 0.1 M HCl. *N*,*N*,*N*′,*N*′ - Tetramethylethylenediamine (0.05 mL), 1 mL of riboflavine (0.5 mg/mL) and 1 mL of ammonium persulfate (1 mg/mL) were added per 100 ml of the gel mixture to initiate and catalyse their polymerization between two day-light fluorescent bulbs over a period of 1 h. The upper catholyte consisted of 80 mM 2-alanine with 10 mM Tris. The lower anode buffer was 0.1 M Tris with 0.02 M acetic acid, and it was used repeatedly while the pH remained over 7.

Electrophoresis in the anodal direction was carried out in an ice-refrigerated Plexiglass apparatus for 120 × 800 × 2 mm vertical gel slabs by applying a pulsed current at 15 mA and 20–30 V/cm until the marker dye, bromophenol blue, reached the gel end (about 2.5–3 h). After electrophoresis, the gels were stained for isozymes by applying standard histochemical methods described by Wendel and Weeden (1989). Electrophoretic isozyme phenotypes (hereafter zymograms) were genetically interpreted as one-banded homozygotes or multiple-banded heterozygotes, with balanced and unbalanced heterozygotes recorded by the differential staining intensity of bands and taking into account the known monomeric versus dimeric structures of enzymes, as described and illustrated in several papers (e.g., López-Pujol et al., 2004, 2007).

2.4. Data analyses

Allozymic genetic diversity among the germinated seedling progeny of populations was estimated by the observed and expected heterozygosities H_o and H_e of Nei (1972), the mean number of alleles at polymorphic loci analysed (A) and the effective number of alleles (A_e) using the TETRAPLOIDE software (Decarli and Leinemann, 2003). Cytological studies have shown that alfalfa has regular meiosis with mostly 16 bivalents and a low number of multivalents (0.6–1.68 per cell) in some individuals, indicating only minor chromatid segregation (McCoy and Bingham, 1988). Therefore, H_e values were calculated assuming chromosomal segregation. Values of the Wright's inbreeding coefficient F were computed from the H_o and H_e estimates according to the equation $F = 1 - (H_o/H_e)$. Heterozygosity at the population level and among the seed progeny was characterized by the proportion of two, three and four-allelic heterozygotes and by the observed heterozygosity (H_o). Allele frequencies at the isozyme loci for populations were provided by TETRAPLOIDE. UPGMA dendrogram of relationships between populations based on the allele frequency data and Nei's standard genetic distances (Nei, 1972) was constructed with the DISPAN software (Ota, 1993). For comparison, UPGMA dendrogram was also constructed based on the D_o genetic distances of Gregorius (1974) between the population pairs provided by TETRAPLOIDE in an input to a sub-program NEIGHBOR of the PHYLIP software (Felsenstein, 2009).

3. Results

Three enzymes, IDH, PGI and PRX, provided three interpretable, polymorphic allozyme loci, *Idh-A*, *Pgi-A* and *Prx-E*, altogether with 15 alleles. Allele numbers ranged from two for *Prx-E* to eight for *Pgi-A*. Both balanced and unbalanced heterozygous isozyme phenotypes were observed among the individuals as characteristic of the autotetraploid. Unbalanced genotypes combining three different alleles of IDH-A and PGI-A were recorded in all populations, but only one genotype combining the four most frequent alleles of *Pgi-A*, A2457, was revealed in five populations, mostly at low frequencies below 5%. The inclusion of thioglycerine instead of cysteine as a sulphydryl protector in the homogenization buffer was needed to develop IDH-A bands of sufficient intensity and to inhibit isoperoxidases otherwise overlapping PRX-E. PGI zymograms displayed a weak, invariant band of a slower PGI-B in a set of individuals, but lacking in many others, presumably because of frequent null alleles. Shikimate dehydrogenase zymograms revealed two isozymes, SKD-A and SKD-B, with two and three allozymes, respectively. The SKD zymograms, however, could not be interpreted as genotypes due to overlapping of two-banded allelic phenotypes and could not be used for the quantitative characterization of genetic diversity by allele frequencies and heterozygosity.

All three isozymes used were highly polymorphic, with IDH-A and PGI-A displaying variation with up to five and eight allozymes, respectively, and PRX-E with two allozymes frequent in all populations and subpopulations. The mean H_e for the isozyme loci when analysed separately varied form 0.752 *Idh-A* and 0.823 for *Prx-E* to 0.928 for *Pgi-A* (data not shown), indicating that all three loci are highly informative.

Average genetic diversity measures for the seven populations are shown in Table 1. Mean number of alleles (A) for three loci ranged from 3.3 to 4.7, being highest in the smallest population P3. Effective number of alleles (A_e) was highest in a small population P5 with $A_e = 2.3$, whereas the largest population P4 and the smallest population P3 had both the lowest $A_e = 2$. The mean Nei's panmictic genetic diversity H_e was quite high, ranging between populations from 0.795 to 0.893. Remarkably, both maximum and minimum H_e values were found for the two small populations, P3 and P5, whereas the largest population P4 has H_e close to that of the smallest population P3. The seed progeny of five populations showed prevalent out-crossing ($F \leq 0.1$), indicating that it is derived from a nearly random bumblebee-mediated mating between the adult mother plants. Only population P7 had a bit higher F, showing about 14 percent inbreed progeny.

Regression analysis revealed no significant correlations between H_e ($p = 0.1$, $R = 0.3$), A ($p = 0.5$, $R = 0.4$), A_e ($p = 0.7$, $R = 0.2$) or F ($p = 0.4$, $R = 0.4$) and population size. The smallest population P3 has equally high genetic diversity $H_e = 0.795$ than the largest population P4 ($H_e = 0.808$) of about hundred times larger, 1.5 and 165 ha respectively. Regression analysis also showed no significant correlation between H_e ($p = 0.5$, $R = 0.4$), A ($p = 0.9$, $R = 0.03$), A_e ($p = 0.3$, $R = 0.5$) or F ($p = 0.8$, $R = 0.09$) and the population isolation distance. All populations and subpopulations studied showed high values of the Nei's genetic diversity measure H_e ranging between 0.795 and 0.893, showing that there is sufficiently space to reveal a loss of genetic diversity from this high H_e level.

Data about the distribution of allele frequencies among three polymorphic isozyme loci in populations studied (Table 2) show that only two of five *Idh-A* alleles, *A3* followed by *A4*, are frequent in all populations, whereas alleles *A1* and *A2* were detected in only one to few individuals in populations P1a, P1b, P2, P3, P4, and P6, being absent in populations P5, P6b and P7. Likewise, only three of eight *Pgi-A* alleles, *A5*, *A5* and *A7*, contribute most to the genetic diversity and its variation, whereas allele *A8* was detected only in the farthest and most isolated Kunda population P7. Both alleles of *Prx-E* are frequent in all populations and contribute significantly to the genetic diversity.

The Nei's standard genetic and D_o genetic distance matrix between populations (Table 3) and respective UPGMA cluster analyses (Fig. 2) reveal only partial correspondence to geographical distances. The populations clustered on the UPGMA trees into two major groups. One group includes nearby populations P1a, P1b, P2, P3 and P4 placed in the same Paldiski region, but population P7 is from a very distant Kunda region, about 170 km away. The Paldiski populations P1a and P1b form a separate branch, which is linked to populations P2–P4 of the same region. Populations P5, P6a and P6b, forming a separate group, are

Table 2
Allele frequencies at three polymorphic loci in seven populations of *Medicago sativa* ssp. *falcata*.

Locus	Allele	Populations								
		P1a	P1b	P2	P3	P4	P5	P6a	P6b	P7
Idh-A	1	0.015	0.014	0.005	0.043	0.037	0	0.007	0	0
	2	0.010	0	0	0.012	0.037	0	0.007	0	0
	3	0.730	0.707	0.641	0.780	0.684	0.593	0.711	0.728	0.688
	4	0.173	0.236	0.293	0.128	0.199	0.352	0.197	0.211	0.258
	5	0.071	0.043	0.060	0.037	0.044	0.056	0.079	0.050	0.053
Pgi-A	1	0	0	0.004	0.004	0	0	0	0	0
	2	0	0.048	0.032	0.022	0.043	0.064	0.011	0.010	0.014
	3	0.013	0.005	0.020	0.022	0.032	0	0.008	0	0
	4	0.174	0.221	0.067	0.127	0.106	0.360	0.462	0.372	0.164
	5	0.482	0.471	0.282	0.246	0.346	0.216	0.163	0.156	0.333
	6	0	0	0	0.011	0	0.004	0.008	0.007	0.014
	7	0.330	0.255	0.595	0.569	0.473	0.356	0.348	0.455	0.460
	8	0	0	0	0	0	0	0	0	0.014
Prx-E	1	0.385	0.375	0.375	0.445	0.270	0.415	0.381	0.473	0.323
	2	0.615	0.625	0.625	0.555	0.730	0.585	0.619	0.527	0.677

adjacent in the Kurkse region, about 15–20 km away from the Paldiski populations. Thus, populations of the Kurkse region and populations of the Paldiski region appear in two separate clusters, indicating geographic structuring in this region.

The data show that genetic differentiation between populations of sickle medic is not strictly associated with the geographical isolation distances. Thus, population P7 of the geographically remote Kunda region was genetically closest to population P4 of the Paldiski region. At the same time, there was a clear geographic pattern in the differentiation between populations in the Kurkse (populations P5, P6a and P6b) regions (Fig. 2).

4. Discussion

The results obtained show that fragmentation of initial, large grasslands of North Estonia into smaller and isolated patches through the anthropogenic habitat destruction during the last century has not yet caused the expected reduction of genetic diversity in small populations of the native sickle medic. No significant association between genetic diversity and population size or isolation distance was found by the regression analysis of the allozyme variation data.

There are contradictory reports in literature about the effect of habitat fragmentation into smaller population patches on the genetic diversity (Young et al., 1996). Consistent with theoretical predictions, many studies have shown that fragmentation has a strong impact on genetic diversity, with smaller populations showing a loss of genetic diversity (reviewed by Ellstrand and Elam, 1993; Leimu et al., 2006; Honnay and Jacquemyn, 2007; Aguilar et al., 2008). However, and consistent with our study, there are also many recent papers reporting no significant correlation between H_e and population size for many other herbaceous perennials like *Scabiosa canescens* and *Scabiosa columbaria* (Waldmann and Andersson, 1998), *Gymnadenia conopsea*, a fragrant orchid (Gustaffson, 2000), *Anthirrhinum charideny* and *Antirrhinum valentinum* (Mateu-Andres and Segarra-Moragues, 2000), *Campanula glomerata* (Bachmann and Hensen, 2007), *Vincetoxicum hirundinaria* (Leimu and Mutikainen, 2005), *Primula elatior* (Jacquemyn et al., 2004), *S. columbaria* (Pluess and Stöcklin, 2004), *Globularia bisnagaria* (Honnay et al., 2007), alpine species *Epilobium fleischeri*, *Geum reptans* and *Campanula thyrsoides* (Kuss et al., 2008), and others.

Some studies have reported a positive statistical correlation between the genetic diversity and population size, but the data presented show no difference in genetic diversity between smallest and largest populations. For example, Van Rossum

Table 3
Matrix of pair-wise differentiation between populations of *Medicago sativa* ssp. *falcata* based on the D_o genetic distances of Gregorius (upper right triangel) and Nei standard genetic distances (lower left triangle).

Population	P1a	P1b	P2	P3	P4	P5	P6a	P6b	P7
P1a	0	0.056	0.146	0.141	0.131	0.163	0.120	0.159	0.106
P1b	0.000	0	0.145	0.177	0.137	0.143	0.132	0.165	0.106
P2	0.049	0.060	0	0.110	0.114	0.142	0.168	0.169	0.092
P3	0.042	0.059	0.019	0	0.136	0.183	0.171	0.127	0.137
P4	0.024	0.031	0.017	0.030	0	0.196	0.179	0.183	0.071
P5	0.057	0.040	0.054	0.064	0.060	0	0.101	0.106	0.144
P6a	0.064	0.055	0.086	0.063	0.071	0.017	0	0.080	0.142
P6b	0.062	0.061	0.056	0.030	0.067	0.019	0.011	0	0.136
P7	0.018	0.021	0.012	0.025	0.004	0.033	0.049	0.042	0

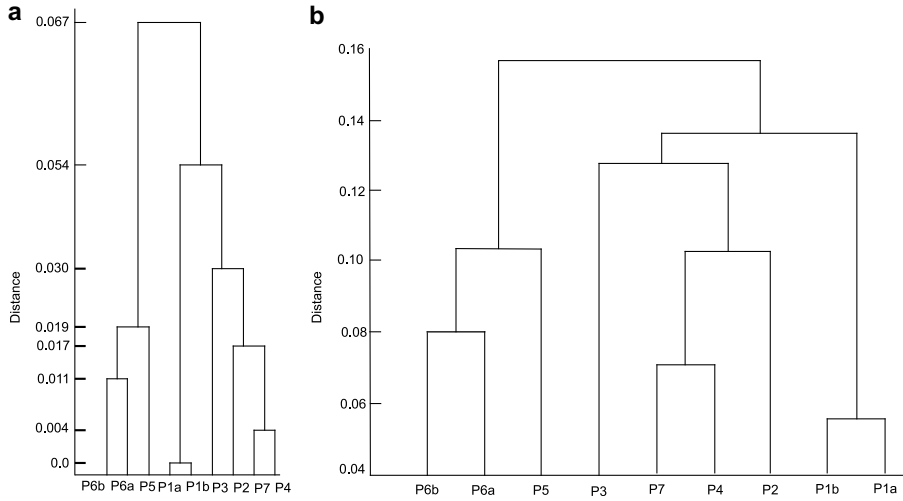

Fig. 2. UPGMA dendrogramms illustrating genetic relationships of seven populations of *Medicago sativa* ssp. *falcata*, based on Nei's standard genetic distances (A) and on D_o genetic distances (B). Numbers at nodes show bootstrap values (1.000 replicates).

and Prentice (2004) found significant positive relationships between population size and allozyme richness A and diversity H_e among a set of 34 Nordic populations of the perennial herb *Silene nutans*, but the data in their Table 2 show that the three largest populations with sizes 800, 1000 and 1200 individuals displayed overlapping A and H_e values with those of smallest populations ranging between 4 and 25 individuals. In an earlier study of Van Rossum et al. (1997), no association between population size and any measure of allozyme diversity was found among 34 Belgian populations of *S. nutans*. Similarly, AFLP genetic diversity measures P and H_e were independent of population size among 15 Belgian populations of *P. elatior* of different age (Jacquemyn et al., 2004).

Several studies have found no correlation between population size and the genetic diversity measure H_e, but population size appeared positively correlated with the proportion of polymorphic loci and the mean observed number of alleles, e.g., Van Treuren et al. (1991) for perennials *Salvia pratensis* and *S. columbaria* inhabiting calcareous grasslands, Raijmann et al. (1994) for *Gentiana pneumonanthe*, Sun (1996) for the orchid *Spiranthes sinensis*, Young et al. (1999) for the endangered grassland perennial *Rutidosis leptorrhynchoides*, Van Rossum et al. (2002) for *P. elatior*. These data show that the number of alleles is more sensitive to the reduction of population size through fragmentation than is the level of heterozygosity, which is consistent with theoretical expectations. In this respect it is notable that we found no correlation between population size and any of the three estimated measures of allozyme genetic diversity, A, A_e or H_e, for the sickle medic populations. Remarkably, the mean number of allozymes was even highest in the smallest population P3. Several alternative explanations for the maintenance of genetic diversity in small habitat fragments are discussed in recent literature reviews (Leimu et al., 2006; Honnay and Jacquemyn, 2007; Aguilar et al., 2008). One possible explanation forwarded is that not enough time has elapsed since the beginning of habitat fragmentation to cause notable decrease of genetic diversity by processes leading to the genetic erosion in small and isolated populations, such as genetic drift and inbreeding. This explanation has been proposed to account a lack of reduced genetic variation in fragmented populations of long-living trees (e.g., Young et al., 1993) and herbaceous perennials (e.g., Rosquist and Prentice, 2000). The effect of habitat fragmentation and population isolation may take years to become evident because long-living perennials may respond slowly to a decline in changing habitat conditions (Ellstrand and Elam, 1993). Given the perennial growth form of sickle medic, it may be plausible to assume that the number of generations grown in fragmented alvar patches of North Estonia formed during the last century was insufficient for processes of genetic drift and inbreeding to reduce the genetic diversity in small populations.

However, the insufficient time elapsed since the onset of extensive destruction and fragmentation of Estonian alvars may not be the sole reason to explain the lack of genetic erosion in small and isolated populations of sickle medic. Thus, a loss of genetic diversity has been observed in small forest fragments of several long-lived trees, e.g, for the dioecious conifer *Halocarpus bidwillii* (Billington, 1991), *Eucalyptus albens* (Prober and Brown, 1994), *Pithecellobium elegans* (Hall et al., 1996), the European beech *Fagus sylvatica* (Jump and Peñuelas, 2006), and for other tree species. And contrary to theoretical expectations, no loss of genetic diversity could be found in small and isolated populations of several annuals, e.g., for *Warea carteri* (Evans et al., 2000), *Cordylanthus palmatus* (Fleishman et al., 2001), *Clarkia dudleyana* (Podolsky, 2001), and biennials, e.g., for *Gentianella austriaca* (Geimler and Dobeš, 2000), *Centaurium erythrea* (2009), *Sabatia angularis* (Spigler et al., 2010). Evidently,

there should be some other reasons that modify the expected relationships between the population size and genetic diversity observed for many species with different lifetimes that should be addressed to explain the results obtained for each particular species studied.

Indeed, there are several other characteristics inherent to sickle medic besides its perennial life form that can contribute to the maintenance of high genetic diversity even in small and isolated populations. In particular, purging from the progeny homozygous individuals of lower fitness formed through the increased inbreeding characteristic of small populations should be considered as a possible factor against genetic erosion of small populations. Our results about high levels of heterozygosity among the viable seedling progeny combined with the literature data about high seed abortion through the early-acting inbreeding depression characteristic of *M.* ssp. *sativa* (e.g., Cooper et al., 1937; Busbice, 1968, and others.) indicate a strong selection acting against inbreed homozygous offspring which is beneficial for the population survival. High level of early inbreeding depression causing the abortion of homozygous genotypes formed by selfing is a plausible mechanism to explain the observed significant genetic diversity at polymorphic isozyme loci by favouring heterozygous genotypes among the seed progeny. In this respect it is remarkable that we detected no increase in the *F* values among the progeny of small populations, except the geographically isolated population P7, contrary to theoretical expectations and empirical evidence reviewed by Leimu et al. (2006) and Honnay and Jacquemyn (2007). Selection against homozygous progeny by the mechanism of embryo abortion may be responsible for a general lack of increased inbreeding coefficient among the seed progeny in small populations of sickle medic (Table 1), as also suggested by Honnay and Jacquemyn (2007). Abortion of self-fertilized embryos, also described in literature as early-acting inbreeding depression or late-acting incompatibility, is widely distributed among outcrossing plants of various taxonomic groups (reviews: Seavey and Bawa, 1986; Charlesworth and Charlesworth, 1987) and is considered as an effective evolutionary mechanism for purging genetic load caused by deleterious alleles exposed to selection in selfed, homozygous seed progeny (Charlesworth and Charlesworth, 1987; Charlesworth, 1989). Numerous studies have shown that plant life history traits, especially the breeding system have a strong impact on the extent and distribution of genetic diversity within and among plant populations (reviewed by Hamrick and Godt, 1996). Fragmentation of previously large grasslands and other habitats into smaller and isolated local populations will lead to changes in the plant breeding system towards increased inbreeding and higher homozygosity with the concomitant loss of genetic diversity and potential viability of plant species in small, remnant populations (reviewed by Ellstrand and Elam, 1993; Young et al., 1996, and others). The ability of *M. sativa* plants to purge defective homozygous alleles from the seed progeny through the inherent mechanism of early-acting inbreeding depression contributes to the maintenance of high levels of genetic diversity in the small and fragmented populations of this species, including ssp. *falcata*. The genetic diversity among the seed progeny is derived from bumblebee mediated crosses between reproductive adult plants and thus reflects the genetic diversity existing in a wild population. The present-day adult population is continuously renewed from the seed bank formed during previous years and thus provides the population historical memory about past genetic diversity and seedling selection (Templeton and Levin, 1979). The every-year new contribution to the seed bank provides continuity to the genetic memory and is an important factor for the maintenance of genetic diversity and acts against the genetic erosion in small and isolated populations. A recent paper of Honnay et al. (2008) reviews studies providing evidence that a persistent seed bank may mitigate the consequences of habitat fragmentation and protect small and isolated plant populations from genetic drift and local extinction. Alfalfa has high percentage of seeds with hard coats impermeable to water, allowing them to remain dormant for years and contributing to formation of a persistent seed bank (e.g., Acharya et al., 1999). We found that scarification was needed to germinate the seeds of sickle medic after six months after collecting and maintaining in a refrigerator, indicating a potential to form a durable seed bank.

Autotetraploid nature of ssp. *falcata* may additionally contribute to maintaining high levels of genetic diversity by higher possible level of individual heterozygosity with up to four different alleles instead of two in diploids. Consistent with theoretical expectations (Bever and Felber, 1992), isozyme studies have shown that autopolyploids display significantly higher levels of heterozygosity than their diploid relatives (e.g., Lumaret, 1982; Soltis and Soltis, 1989; Brown and Young, 2000; Hardy and Vekemans, 2001, and others). In addition, autopolyploidy slows down the loss of heterozygosity at neutral and nearly neutral loci through inbreeding in small populations, simultaneously allowing elimination of recessive lethals and highly deleterious alleles from the seed progeny through the early-acting inbreeding depression, as shown for autotetraploid alfalfa (*local cited*). Thus, the autotetraploid nature, combined with the early-acting inbreeding depression and every-year submission of highly heterozygous seed progeny to the seed bank are plausible factors contributing to the maintenance of high genetic diversity in small and isolated populations of sickle medic in fragmented Estonian alvars. However, polyploidy alone, without inherent early-acting inbreeding depression, may be insufficient for preventing genetic erosion, as shown for fragmented populations of a tetraploid pea *Swainsona recta* (Buza et al., 2000) and for isolated populations of different sizes of the autotetraploid Pyrenean endemic *Delphinium montaneum* (López-Pujol et al., 2007).

A notable result of the study is that genetic differentiation of populations into two major groups (Fig. 2) was only partly associated with the geographical placement of populations because population P7 of the geographically remote Kunda region was genetically closest to population P4 of the Paldiski region. Otherwise, there was a clear geographic pattern in the differentiation of populations into groups of the Kurkse and Paldiski regions.

Another notable result is that genetic differentiation of populations into the two major groups appears to be associated to the soil conditions. The Paldiski and Kunda populations of the first major group are growing on nutrient poor light soils on a limestone bed with a soil depth less than 20 cm. The Kurkse populations P5 and P6 of the second group are also situated on light dry soils, but are more nutritious and with deeper humus depth over 20 cm, suitable for agricultural land use. The

potential association of genetic differentiation of populations with the soil conditions is of interest and will deserve further study.

To summarize, the analysis of genetic diversity in small and isolated populations of ssp. *falcata* among the seed progeny has allowed us to propose that a combination of specific biological features such as early-acting inbreeding depression and continuously renewing seed bank combined with the autotetraploid nature and perennial life form may be crucial for the maintenance of genetic diversity in small and isolated populations of sickle medic. Our future research will assess genetic diversity and local population structure in remnant sickle medic populations at the adult life stage in order to check the conclusions made in the present study on the basis of analyzing the seed progeny. This may reveal additional important evidence to explain the maintenance of genetic diversity in small and isolated populations of sickle medic. In particular, population structure and potential clonality of sickle medic, which could not be revealed by analyzing the seed progeny, may have a strong impact and will be addressed.

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Highly variable clonal diversity and spatial structure in populations of sickle medic

Karin Kaljund*, Malle Leht, Vello Jaaska

Department of Botany, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, 5 Kreutzwaldi str., 51014 Tartu, Estonia

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ABSTRACT

Clonal diversity and spatial structure in six remnant Estonian populations of sickle medic *Medicago sativa* ssp. *falcata* growing in different habitat conditions were estimated at three different spatial scales, in 1 m^2 and 4 m^2 small-scale plots and in 30–60 m long linear transects, using four polymorphic isozyme markers. Sampling all ramets in fourteen 1 m^2 quadrates yielded 419 ramets which displayed 113 multilocus genotypes (MLGs), whereas 311 ramets from 4 m^2 plots exhibited 144 genets and 262 ramets in six transects revealed 223 genets. All MLGs except two were distinct, indicating extremely high genotypic diversity and strong differentiation among populations. Highly variable small-scale spatial structure with adjacent ramets of identical MLGs and different sizes was detected in quadrates up to monoclonality of some plots. Differences in local disturbances and land-use history of populations were found to be associated with their variable genotypic structure and diversity. Sexual reproduction was locally suppressed in abandoned grassland at places with a dense cover of competing grasses and thick litter layer, however, still occurring in suitable or disturbed places. Strong competition at sites densely covered with adult plants has led to the local elimination of small clones and preferential spread of survived clones. High clonal richness observed in long transects with up to 100% consisting of unique MLGs suggests ability of sickle medic for repeated sexual reproduction in established populations. Local disturbances are supposed to provide suitable microsites for seedling recruitment, evidenced by the presence of unique and small MLGs of recent origin.

1. Introduction

Plant populations are characterized by highly variable levels of local genetic differentiation and structuring at different scales depending on habitat conditions, species-specific life history traits, especially on the dispersal ability and mating system (Hamrick et al., 1979; Loveless and Hamrick, 1984; Hamrick and Godt, 1996). The ability to reproduce vegetatively is one of the life history traits that has a strong impact on the formation of spatial patterns of individual genotypes within populations (Ellstrand and Roose, 1987; Honnay and Jacquemyn, 2008; Silvertown, 2008). The genetic diversity and formation of local genetic structure in populations of clonal species is shown to result from the interplay between sexual reproduction through seeds and subsequent vegetative spread, revealing the importance of sexual recruitment of new genotypes for the maintenance of genetic diversity (Widén et al., 1994; Honnay and Jacquemyn, 2008; Silvertown, 2008). The relative contribution of sexual and vegetative reproduction was found to vary widely within species depending on the habitat conditions of populations, up to loss of sexual reproduction (Eckert, 2001; Silvertown, 2008). Comparative studies of species with different

* Corresponding author. Tel.: +372 7311 874; fax: +372 7383 013.
E-mail addresses: karin.kaljund@emu.ee (K. Kaljund), malle.leht@emu.ee (M. Leht), vello.jaaska@emu.ee (V. Jaaska).

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life histories growing in various habitats will help to understand factors shaping the genotypic structure and diversity of plant populations.

The general goal of this work was to evaluate genotypic diversity and spatial structure among and within Estonian sickle medic populations growing in different habitat conditions. The following specific questions were addressed: (i) How does clonal diversity vary within and among sickle medic populations depending on the sampling scale? (ii) How are genets spatially distributed in small-scale 1 m^2, medium-scale 4 m^2 squares and large-scale transects? (iii) How large and variable are the sickle medic clone sizes? (iv) How are the clonal structure and diversity associated with the ecological conditions and spatial heterogeneity of populations?

2. Materials and methods

2.1. Study species

Medicago sativa L. ssp. *falcata* (L.) Arcangeli (sickle medic, hereafter *falcata*) is a perennial herbaceous legume that reproduces both sexually by seeds and vegetatively by rhizomes (Lesins and Lesins, 1979). It belongs to the *M. sativa* s.l. species complex together with *M. sativa* ssp. *sativa* (alfalfa or lucerne, hereafter *sativa*). The taxonomy of the group remains controversial because of different circumscriptions in different taxonomical treatments, mostly either at the level of subspecies (e.g. Ball, 1968; Small and Brookes, 1984) or distinct species (e.g. Lesins and Lesins, 1979). Subspecies *sativa* and *falcata* are clearly distinguishable by the flower color (blue-purple versus yellow) and pod shape (a spiral of 2–3 turns versus falcate). However, in adjacent and sympatric populations they hybridize readily forming a morphological continuum of fully fertile hybrids treated as *M. sativa* ssp. *varia* (Martyn.) Arcangeli.

During our recent field work we found that of 106 populations examined only 15 were pure *falcata* populations, indicating extensive hybridization between native *falcata* and introduced *sativa* in Estonia (Kaljund and Leht, in press).

2.2. Study sites, populations and sampling

The study was carried out in six natural populations of pure *falcata* situated in coastal regions of North and West Estonia, mostly on remnant fragments of natural and semi-natural calcareous grasslands (alvars) of different habitat conditions and history (Fig. 1). Population P1 is a former large pasture situated on island Vormsi in West Estonia. Populations P2, P4 and P5 of *falcata* in West and North Estonia region have survived mainly because they are situated in a former Soviet military zone closed to civilians from 1940 until 1995, without fields of cultivated *sativa* or *varia* nearby, thus avoiding introgression from cultivated plants. Population P3 is a small abandoned grassland isolated from other *falcata* populations by unsuitable habitats such as forests and roads. Population P6 is situated directly at the coast of Finnish Gulf in North Estonia on an overgrown sand dune of a public beach.

The upper 5–7 cm parts of shoots with younger leaves were collected from all shoots (ramets) grown directly from soil in two to three small-scale 1 m^2 squares per population from six different local populations. In order to determine the extent of clonal spread at a medium scale, a set of 35 ramets were also sampled near the nodes of 35 equal 20 × 20 cm grids of three 4 m^2 squares. Linear transects of 30–60 m long from populations P2, P3, P4 and P5 were also analyzed by sampling ramets spaced sequentially at distances of 0.7–1.5 m from each other in order to evaluate genotypic diversity at a larger spatial scale. Within the study squares and transects, ramets were mapped and analyzed for the spatial distribution of four-locus allozyme genotypes and the clonal diversity indices.

Fig. 1. Map of the six sampling sites of sickle medic *Medicago sativa* ssp. *falcata* in North and West Estonia.

2.3. Electrophoretic allozyme analyses

Among nine enzymes tested in preliminary analyses, the following four isozymes proved sufficiently polymorphic and had genetically interpretable electrophoretic phenotypes with distinct allozyme bands suitable for the delimitation of allozyme-based multilocus genotypes: IDH-A (isocitrate dehydrogenase, EC 1.1.1.42), PGI-A (phosphoglucoisomerase, EC 5.3.1.9), PRX-E (peroxidase, EC 1.11.1.7) and LAP-A (leucine aminopeptidase, EC 3.4.11.1).

Electrophoretic analyzes were performed as described in Kaljund and Jaaska (2010). IDH and PGI isozymes were stained as described by Wendel and Weeden (1989) with minor modifications. For peroxidase staining, gels were first incubated for 20 min at 35 °C in 0.2 M acetate buffer of pH 4.8 containing 1 mM pyrocatechine substrate coupled with 1 mM *o*-diansidine and 1.2% oxalic acid, followed by adding H_2O_2 up to final 0.01%. For LAP, gels were incubated for 20 min 35 °C in 0.2 M maleate buffer of pH 5.6 containing 1 mg/ml L-leucyl-2-naphthylamide hydrochloride substrate, 4 mM $MnCl_2$ and 0.1% detergent Triton X-100, followed by adding about 10 mg of the diazo dye Fast Black K (Sigma) dissolved in 0.2 ml *N*,*N*-dimethyl formamide.

Electrophoretic isozyme phenotypes (hereafter zymograms) were genetically interpreted as one-banded homozygotes or multiple-banded heterozygotes with two, three or four allozyme bands and taking into account the monomeric versus dimeric structures of enzymes. Balanced and unbalanced heterozygotes were recorded by the differential staining intensity of bands on heterozygous zymograms, consistent with the autotetraploid nature of alfalfa (Quiros, 1982).

2.4. Data analyses

For the quantitative characterization of genotypic diversity within quadrates and transects, we determined genotypic richness as $R = (G-1)/(N-1)$, where G is the number of genotypes and N is the number of sampled ramets in a plot (Dorken and Eckert, 2001). Each distinct multilocus genotype based on four isozyme loci is assumed to correspond to a separate clone, and thus the genotypic richness is taken as a measure of clonal diversity. Three additional measures of clonal diversity in the study plots, the Simpson's index D_s, the effective number of genotypes G_e and the evenness of the effective number of genotypes E were calculated with the use of the software GENOTYPE and GENODIVE of Meirmans and Van Tienderen (2004). D_s values range from zero in a population composed of a single genotype to 1.0 when every individual sampled has different genotype. The GENOTYPE program allows choose a threshold value in order to lump together closely related genotypes. Given that isozymes are evolutionarily relatively conservative molecular markers which frequently tend to underestimate the actual number of multilocus genotypes and clonality in species with a low effective allozyme number and polymorphic isozymes (Widén et al., 1994), we applied a zero threshold value for our data. To evaluate the statistical power of the four isozyme loci used for the discrimination of genotypes, a total number of possible genotypes in an autotetraploid across the four in the studied quadrates and transects (G_p) was calculated according to Trapnell et al. (2011) as

$$G_p = \prod_{i=4}^{n=4} {}^{*}g_i$$

where g_i is the number of genotypes at the ith locus and n is the number of loci ($n = 4$). The number of possible genotypes at each autoteraploid locus $g_i = 1/24(g)(g+1)(g+2)(g+3)$, where g is the number of alleles at a locus (Haldane, 1948). Values of G_p were calculated for each study plot using the effective number of alleles at each isozyme locus in the respective plot calculated with the TETRAPLOIDE software (Decarli and Leineman, 2003).

3. Results

The data on the genotypic diversity measures at the three spatial scales, 1 m^2, 4 m^2 and transects, are summarized in Table 1. The four polymorphic allozyme loci allowed to discriminate all or most of the ramets as unique MLGs in transects (Table 1), reflecting high discriminating power of the allozyme loci used enabling to attribute ramets to shared allozyme genotypes which are thus assumed to represent putative genets. The total number of alleles per locus were five, seven, two and five for Idh-A, Pgi-A, Prx-E, and Lap-A, respectively. The number of possible genotypes was always much higher than the number of genotypes found (Table 1), so indicating that distinct MLGs detected may be attributed to different genets.

3.1. Genotypic diversity and spatial structure within 1 m^2 small-scale quadrates

Sampling all ramets in fourteen 1 m^2 quadrates yielded 419 ramets which displayed 112 MLGs, i.e. 27% of ramets had different MLGs. Only one shared MLG was found in two geographically distant populations P2 and P5. Otherwise, each population displayed a unique set of MLGs, indicating high overall genotypic diversity. The indexes of genotypic diversity were highly variable among populations and between quadrates within populations (Table 1). Genotypic richness (R) varied remarkably among the 14 analyzed plots, ranging from 0 to 0.88. The variation was most contrasting in P4 were in plot Q1b genotypic richness was 0.88, but in plots Q1a and Q1c it was only 0.03.

Most of the 1 m^2 sites displayed highly variable spatial structure: the number of ramets in the fourteen quadrates varied from 14 to 56. Ramets were spatially mostly aggregated (clumped) into MLGs of different sizes, but in some plots genets were

Table 1
Summary of genotypic diversity parameters for the squares and transects in populations of sickle medic; P, population; Q1, 1 m^2 quadrate, Q4, 4 m^2 quadrate; T, transect; *N*, number of ramets genotyped; *G*, number of multilocus genotypes (genets) detected; $R = (G-1)/(N-1)$, genotypic richness; G_e, effective number of genotypes; *E*, the evenness index; D_s, Simpson's index of genotypic diversity; G_p, a total number of possible genotypes across loci, based on the effective allele number at each isozyme locus in a sample.

P; Q; T	*N*	*G*	*R*	G_e	*E*	D_s	G_p
Vormsi P1							
Q1a	56	11	0.18	5.03	0.46	0.82	408
Q1b	34	13	0.36	9.17	0.71	0.92	4434
Haapsalu P2							
Q1a	23	5	0.18	1.77	0.35	0.45	104
Q1b	23	9	0.36	4.68	0.52	0.82	242
Q1c	34	12	0.33	4.21	0.36	0.79	728
T1	38	37	0.97	36.10	0.98	1.00	1670
Kurkse P3							
Q1a	14	1	0.00	1.00	1.00	0.00	54
Q1b	31	1	0.00	1.00	1.00	0.00	1677
Q4a	32	7	0.19	3.32	0.47	0.72	837
Q4b	35	7	0.18	2.72	0.39	0.65	724
Q4c	34	1	0.00	1.00	1.00	0.00	206
T1	59	33	0.55	21.9	0.66	0.97	1651
Paldiski P4							
Q1a	36	2	0.03	1.99	1.00	0.51	130
Q1b	34	30	0.88	26.3	0.88	0.99	3369
Q1c	38	2	0.03	1.11	0.56	0.10	47
Q4a	35	32	0.91	29.8	0.93	0.99	5737
Q4b	35	17	0.47	8.22	0.48	0.90	1119
Q4c	35	10	0.26	4.59	0.46	0.81	1944
T1	40	40	1.00	40.0	1.00	1.00	5810
T2	50	40	0.80	29.1	0.73	0.99	1571
Paldiski P5							
Q1a	14	2	0.08	1.15	0.58	0.14	141
Q1b	27	6	0.19	4.26	0.71	0.79	172
Q4a	35	11	0.29	10.5	0.70	0.93	1734
Q4b	35	28	0.79	21.5	0.77	0.98	555
Q4c	35	27	0.76	23.1	0.86	0.98	5036
T1	35	35	1.00	35.0	1.00	1.00	4385
T2	40	39	0.97	38.0	0.98	1.00	11,670
Kunda P6							
Q1a	25	7	0.25	2.78	0.40	0.67	624
Q1b	30	12	0.38	7.38	0.61	0.89	4579

intermingled. The number of genets in quadrates within populations varied broadly, ranging from 1 to 30. The spatial structure of MLGs in a selected set of six 1 m^2 quadrates of three populations is shown in Fig. 2 in order to illustrate the extreme cases of variation among and within populations. Q1a of P1 comprised 59 ramets belonging to 11 MLGs (Table 1), with a number of ramets in a genet ranging from 1 to 15 (Fig. 2). Two genotypes, 5 and 6, were dominating over others. Q1b of P1 contained 34 ramets of 13 MLGs (Table 1), but the number of ramets in a genet ranged from 1 to only 7. The maximum distance between farthest ramets in a genet of the two quadrates was 0.75 and 0.4 m, respectively, reflecting maximum genet sizes. In both quadrates, some MLGs showed a clear spatial aggregation, whereas others revealed intermingling. P3 was exceptional because the two quadrates examined, Q1a and Q1b, were monoclonal consisting of two different genotypes with 14 and 31 ramets, respectively. The maximum genet size in the two quadrates of P3 was about 1 m.

P4 showed remarkably extreme variation between the two 1 m^2 plots shown in Fig. 2. Thus, Q1a of P4 had only two different genotypes which were distributed intermingled within the plot. The maximum genet size in Q1a and Q1b was 1 and 0.15 m, respectively. The number of ramets in the two genets of Q1a was almost equal: 17 and 19 ramets. In a sharp contrast, in Q1b of P4 nearly all ramets had different genotypes. Only three genotypes in Q1b revealed clumped spacing of nearby growing ramets formed through the clonal growth, while the reminder ramets belong to unique genotypes that have not yet propagated vegetatively (Fig. 2). This indicates that a local disturbance in Q1b accompanied with the increased sexual reproduction from seeds has occurred. We suppose that unique MLGs reflect genets derived through sexual reproduction from seeds, whereas adjacent ramets of 2–4 identical MLGs belong to small genets that have recently started to spread clonally.

3.2. Genotypic diversity and spatial structure within 4 m^2 medium-scale quadrates

In each quadrate one ramet from 35 equal subplots was analyzed. A total of 144 MLGs were detected among 311 ramets in nine quadrates of three populations, i.e. 46% ramets had different MLGs. None of the MLGs was found in more than one population, and all quadrates had unique multilocus genotypes. The number of genets in plots varied from 1 to 32. The indexes of genotypic diversity were variable both among and within P3, P4 and P5 (Table 1). *R* varied notably among the three populations and among nine 4 m^2 plots ranging from 0 up to 0.91.

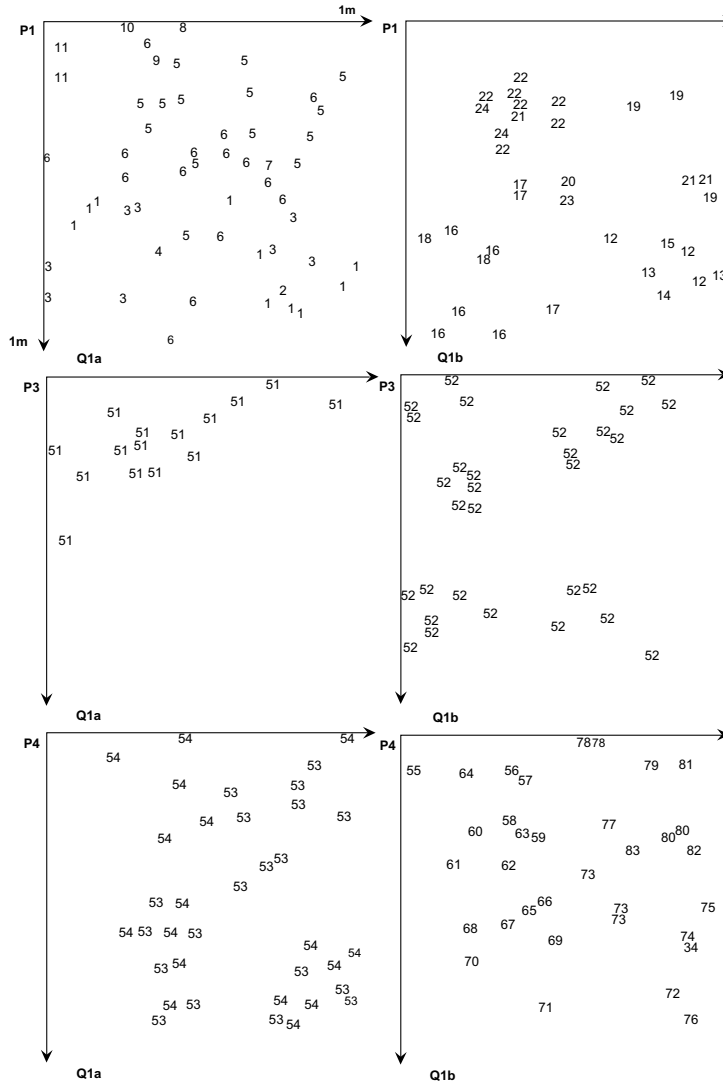

Fig. 2. Spatial distribution of multilocus genotypes in 1 × 1 m quadrates with all ramets sampled and mapped.

The 4 m^2 quadrates showed large variations also in the spatial structure and sizes of genets (Fig. 3). Clonality was the highest in P3. In two quadrates, Q4a and Q4c of P3, the genotypes were spatially aggregated and the maximum genet size was 2 m. In Q4a of P3 the number of ramets per genet varied from 1 to 16, and one genotype was dominating, while in Q4c only one genotype with 34 ramets was found. P4 showed contrasting results with lowest clonality in Q4a and highest in Q4c. In Q4a of P4 the genets were spatially aggregated, the maximum genet size was 0.3 m and the number of ramets in a genet was 1–2. In Q4c the maximum genet size was 1.68 m, the number of ramets in a genet ranged from 1 to 11 and two genets, 177 and 180, were dominant. In Q4a and Q4b of P5 the maximum genet size was 1.15 m and the maximum number of ramets per genet was the lowest, ranging up to 4–5. Genets were spatially aggregated and no dominating genets were observed.

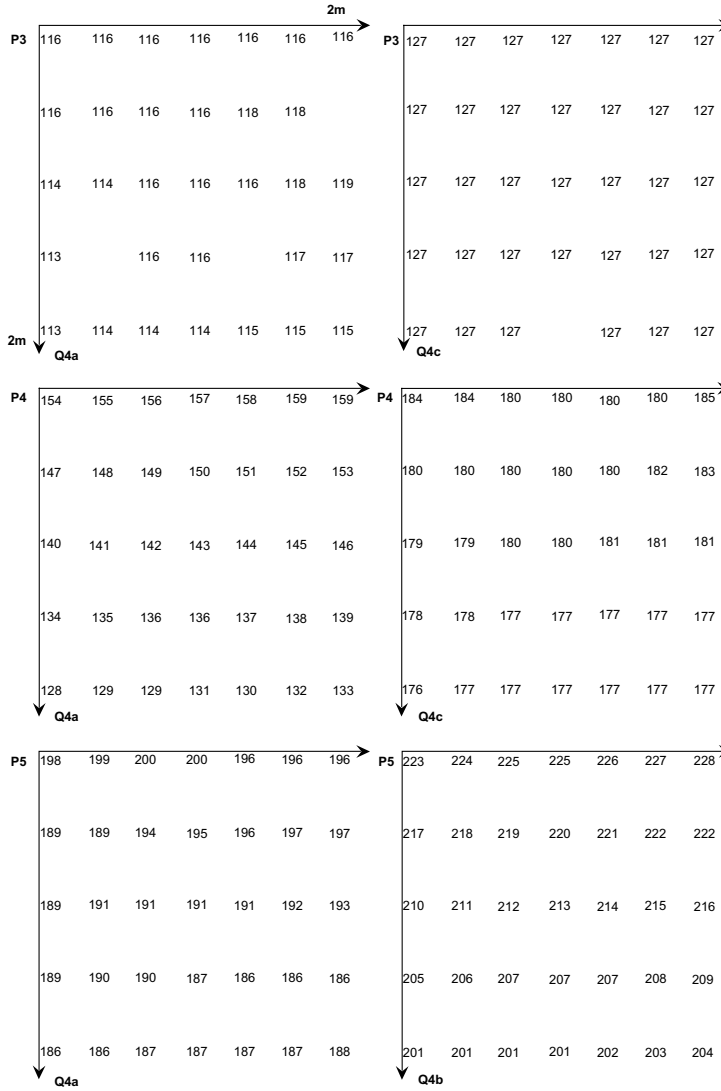

Fig. 3. Spatial distribution of multilocus genotypes in 2 × 2 m quadrates. Empty places represent missing ramets.

3.3. Genotypic diversity and spatial structure within large-scale transects

The long linear transects displayed higher genotypic diversity than small quadrates (Table 1). The number of MLGs detected reached 223 among the total 262 ramets analyzed. The proportion of MLGs has thus increased from 27% in 1 m^2 quadrates to 85% in transects. Only one shared genotype was found: in P4 and P5 two individuals had the same MLG. Clonality along the 60 m long linear transect of P3 was highest ($R = 0.55$), whereas transect T1 of P4 and transects T1 and T2 of P5 revealed no clonality ($R = 1.0$), despite that considerable clonality was observed in most small- and medium-scale quadrates of P4 and P5. This shows that performing analyses at different and appropriate spatial scales is needed in

order to obtain reliable information about the extent of clonality and its variation within and among populations. The number of genets per ramet in the transect of P3 ranged from 1 to 7, the maximum genet size was about 6 m, and 18 ramets out of 59 analyzed had unique genotypes. In P4, transect T2 also revealed moderate clonality ($R = 0.80$), contrary to transect T1 were all sampled ramets had unique genotypes and thus no signs of clonality. These data show that extensive large-scale sampling of adult plants of a clonal species from ecologically different sites of a large population such as P4 is needed to obtain reliable data on the population-level genotypic diversity.

4. Discussion

The most impressive result of the study is that genotypic diversity and spatial structure vary largely both between and within populations of sickle medic with highly variable genotypic richness and evenness among neighboring 1 m^2 and 4 m^2 plots within one and the same population. This raises a question about possible causes of the observed large differences. Although most authors have found high levels of genetic diversity in clonal species at the species level, have some authors recorded quite large variation in the genotypic diversity between neighboring populations of the same clonal species, for example in populations of *Festuca rubra* (Rhebergen et al., 1988) and *Fragaria virginiana* (Wilk et al., 2009). The other extreme was that no genotypic allozyme and RAPD variation was detected within and among numerous populations of the invasive clonal species *Fallopia japonica* throughout Europe (Hollingsworth and Bailey, 2000; Mandak et al., 2005; Krebs et al., 2010). Different authors suggest that the spatial genotypic structure and clonal diversity of populations are largely shaped by relative contribution from sexual reproduction and vegetative growth depending on the ecological conditions, especially on the availability of suitable sites for the seedling recruitment from the seed pool which may vary both between and among populations (Eriksson, 1989; Eriksson and Eriksson, 1997; Honnay and Bossuyt, 2005).

The six *falcata* populations studied differ in their habitat conditions, thus allowing to make inferences about associations between the clonality measures and particular ecological conditions of the populations. Among these differences, vegetation density seems to be especially important ecological factor shaping the relative contribution of sexual reproduction from seeds and subsequent vegetative spread of genets. Population P3 is a small grassland, abandoned about fifteen years ago. It differs from others by dense vegetation with dominating high grasses and thick litter layer suppressing sexual reproduction through the seedling recruitment. Our data suggest that such habitat is associated with the existence of monoclonal quadrates with low or no clonal diversity in P3. We suppose that the most likely explanation of the locally reduced genotypic richness in P3 is that clonal competition has eliminated less fit genotypes and has favored vegetative spread of genotypes adapted to the dense vegetation. Maturing and aging of the population has presumably favored selection of locally superior clones with enhanced vegetative growth. Decline in the genotypic richness and concomitant increase in the clonal reproduction with the population aging and the establishment of dense vegetation cover has been reported in several studies of different species (Scheepens et al., 2007; Silvertown, 2008). The presence of monoclonal quadrates shows that seedling recruitment may be locally even completely suppressed in sites with a dense vegetation cover and can occur only in places of occasional micro-disturbances. Evidently, local disturbances are needed to provide open microsites for safe seedling recruitment in places with a thick litter layer and dense vegetation, characteristic of P1, P3 and some regions of P2, P4 and P5, where quadrates with high or moderate genotypic richness were found. Similarly, P6 is a small population situated on a sandy beach, where trampling of people may have caused small local disturbances associated with a moderate genotypic diversity in 1 m^2 quadrates.

Populations established greatly through sexual reproduction from seeds are expected to consist of a large number of randomly distributed genotypes (Ellstrand and Roose, 1987). Indeed, the genotypic richness reached 0.55 in a large linear transect extending diagonally through the whole P3, indicating input of new genets by sexual reproduction at local microsites of this population. Similarly, 1 m^2 and 4 m^2 plots with high level of genotypic richness were found in populations P4 and P5, evidencing significant level of sexual reproduction in some small sites within these populations. In this respect, large differences among the three 1 m^2 plots of P4 are of special interest, because they were sampled in a region with a uniformly dense cover of sickle medic plants.

Kaljund and Jaaska (2010) proposed that specific biological features of sickle medic, such as early-acting inbreed depression, annually renewing seed bank, autotetraploid nature and perennial life form, might be crucial for the maintenance of genetic diversity in fragmented, small populations. Current data on the similarly high genotypic diversity in transects and some quadrates suggest repeated seedling recruitment. Therefore, we assume that sexual reproduction from seeds at disturbed sites combined with clonality additionally contributes to the maintenance of high genetic and genotypic diversity in populations of sickle medic.

In summary, Estonian populations of sickle medic display wide variation in the genotypic richness and spatial clonal structure. Sickle medic is able to establish itself sexually from seeds in natural grasslands, but the degree of establishment estimated by the genotypic diversity varies widely depending on the vegetation cover and disturbances. Local disturbance events in stable populations with dense vegetation provide opportunities for occasional sexual reproduction from the seed bank and annual seed rain. Variable environmental conditions and different ecological history of populations are associated with the observed high variation in the genotypic diversity and clonal structure within and among the sickle medic populations.

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CURRICULUM VITAE

First name:	Karin
Last name:	Kaljund
Date of Birth:	21.07.1982
Institution:	Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, Department of Botany, 5 Kreutzwaldi Street, Tartu 51014, Estonia
E-mail:	karin.kaljund@emu.ee

Education:

2007-2013	PhD studies in Botany, Estonian University of Life Sciences
2005-2007	MSc studies in Botany and Mycology, Estonian University of Life Sciences
2001-2005	BSc studies in Natural Resource Management, Estonian Agricultural University
1989-2001	Tartu Raatuse Gymnasium

Languages spoken:

Estonian, English, German, Russian

Professional employment:

Since 2007	Estonian University of Life Sciences, Institute of Agriculture and Environmental Sciences, specialist

Research interest:

Botany, population biology, population genetics, ecology

Professional training:

2011	graduate course „7th International School of Conservation Biology," Rovinj, Croatia
2009	PhD course „Molecular marker analysis of plant population structure and processes," University of Copenhagen, Denmark

ELULOOKIRJELDUS

Eesnimi:	Karin
Perekonnanimi:	Kaljund
Sünniaeg:	21.07.1982
Töökoht:	Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, botaanika osakond, Kreutzwaldi 5, Tartu 51014
E-post:	karin.kaljund@emu.ee

Haridus:

2007-2013	doktoriõpe botaanika erialal, Eesti Maaülikool
2005-2007	magistriõpe botaanika ja mükoloogia erialal, Eesti Maaülikool
2001-2005	bakalaureuseõpe loodusvarade kasutamise ja kaitse erialal, Eesti Põllumajandusülikool
1989-2001	Tartu Raatuse Gümnaasium

Keelteoskus:

eesti, inglise, saksa, vene

Teenistuskäik:

Alates 2007	Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, spetsialist

Teadustöö põhisuunad:

botaanika, populatsioonibioloogia, populatsioonigeneetika, ökoloogia

Erialane täiendamine:

2011	kraadiõppurite kursus „7th International School of Conservation Biology," Rovinj, Horvaatia
2009	doktorikursus „Molecular marker analysis of plant population structure and processes," Kopenhaageni Ülikool, Taani

LIST OF PUBLICATIONS

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